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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 5 :
C12N 15/12, C12P 21/02, C07K 13/00, C12P 21/08, A61K 37/02, G01N 33/68, C12N 1/21, 5/10, A61K 39/00, C12Q 1/68

A2

(11) International Publication Number: WO 94/26891

(43) International Publication Date: 24 November 1994 (24.11.94)

(21) International Application Number: PCT/US94/05150

(22) International Filing Date: 18 May 1994 (18.05.94)

(30) Priority Data:

08/065,231	19 May 1993 (19.05.93)	US
08/089,263	7 July 1993 (07.07.93)	US
08/092,549	16 July 1993 (16.07.93)	US
08/106,340	13 August 1993 (13.08.93)	US
08/112,391	24 August 1993 (24.08.93)	US
08/155,111	19 November 1993 (19.11.93)	US
08/162,413	3 December 1993 (03.12.93)	US

(60) Parent Applications or Grants
(63) Related by Continuation

US	08/155,111 (CIP)
Filed on	19 November 1993 (19.11.93)
US	08/162,413 (CIP)
Filed on	3 December 1993 (03.12.93)
US	08/112,391 (CIP)
Filed on	24 August 1993 (24.08.93)
US	08/092,549 (CIP)
Filed on	16 July 1993 (16.07.93)
US	08/106,340 (CIP)
Filed on	13 August 1993 (13.08.93)
US	08/089,263 (CIP)
Filed on	7 July 1993 (07.07.93)
US	08/065,231 (CIP)
Filed on	19 May 1993 (19.05.93)

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(81) Designated States: AU, BB, BG, BR, BY, CA, CN, CZ, FI, GE, HU, JP, KG, KR, KZ, LK, LV, MD, MG, MN, MW, NO, NZ, PL, RO, RU, SD, SI, SK, TJ, TT, UA, US, UZ, VN, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).

Published
Without international search report and to be republished

(54) Title: PURIFIED MAMMALIAN FLT3 LIGANDS AND AGONISTS AND ANTAGONISTS THEREOF

(57) Abstract

This invention provides mammalian Flt3 ligands and fragments thereof, nucleic acids encoding the same, recombinant vectors and host cells comprising such nucleic acids, and antibodies or binding fragments thereof which specifically bind to the ligands or fragments thereof, kits, and pharmaceutical compositions for modifying the biological activity of cells bearing Flt3 ligand receptors. Methods for making and using the foregoing materials are also provided by this invention.

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PURIFIED MAMMALIAN FLT3 LIGANDS AND AGONISTS AND ANTAGONISTS THEREOF

The present invention relates to compositions which function in controlling development and differentiation of mammalian cells, e.g., cells of a mammalian immune system. In particular, it provides proteins and mimetics which regulate development, differentiation and function of various cell types, including hematopoietic cells.

BACKGROUND OF THE INVENTION

Protein tyrosine kinases often play important roles in signal transduction leading to cellular proliferation. The large family of protein tyrosine kinases includes many growth factor receptors. See, e.g., Pawson, *et al.* (1990) *Trends in Genetics 6*:350-356. Growth factor receptors are important in control and regulation of cellular physiology and development. Although these receptors have been found on various cell lineages, their specific roles in regulation of development of different cell lineages are generally poorly understood. Suggestions of a role for a protein tyrosine kinase in hematopoiesis have been largely based upon the identification of the c-kit receptor tyrosine kinase as the W locus, mutations in which affect erythroid and mast cell lineages. See, e.g., Chabot, *et al.* (1988) *Nature 335*:88-89; and Geissler, *et al.* (1988) *Cell 55*:185-192.

Besides the gene product of the W locus, another protein tyrosine kinase had been isolated and characterized. See Matthews, *et al.* (1991) *Cell 65*:1143-1152; and Rosnet, *et al.* (1991) *Oncogene 6*:1641-1650. This protein has been designated Fms-like tyrosine kinase 3 (Flt3) or Flk2. Although it has been localized to particular cell types, e.g., placenta,

gonad, neural, and hematopoietic, its biological effects on cell differentiation and physiology have not been fully described.

Moreover, the receptor should mediate cellular signal transduction in response to a natural ligand. The nature of the ligand has yet to be identified, and its physiological effects and cell lineage specificity remain largely unknown. The distribution of the receptor, however, suggests that the ligand has a role in regulating cell physiology and development in a multiplicity of cell lineages.

There thus is a need to know about the structural, biological, and physiological properties of the regulatory factors which naturally bind to the Flt3 tyrosine kinase receptor.

SUMMARY OF THE INVENTION

The present invention, which is based in part upon the discovery of natural ligands for the Flt3 tyrosine kinase receptor, fills the foregoing need. This invention provides agonists and antagonists of the natural ligands, e.g., mutations (muteins) of the natural sequences, fusion proteins, chemical mimetics, antibodies, and other structural or functional analogs. It also provides isolated nucleic acids encoding proteins of the invention. Various uses of these different protein or nucleic acid compositions are also provided.

The present invention provides substantially pure Flt3 ligands or peptides thereof, or fusion proteins comprising the Flt3 ligand sequence; antibodies specific for binding to a Flt3 ligand; and a nucleic acid encoding a Flt3 ligand or fragment thereof.

The Flt3 ligand or peptides thereof, which can be from a warm blooded animal such as a bird or mammal, including a mouse, comprise at least one polypeptide sequence shown in Table 1. They further may exhibit a post-translational modification pattern distinct from natural Flt3 ligand, including at least one of the features disclosed in Table 2, or

they may induce Flt3 receptors to self-phosphorylate. A further embodiment is a composition comprising such a ligand and a pharmaceutically acceptable carrier.

In antibody embodiments, the antigen can be a mammalian protein, including a mouse; the antibody is raised against a peptide sequence of Table 1; the antibody is a monoclonal antibody; or the antibody is labeled.

In nucleic acid embodiments, the nucleic acid can comprise a sequence of Table 3.

The invention also embraces a kit comprising a substantially pure Flt3 ligand or fragment, e.g., as a positive control; an antibody or receptor which specifically binds a Flt3 ligand; or a nucleic acid encoding a Flt3 ligand or peptide.

The availability of these reagents also provides methods of modulating physiology or development of a cell comprising contacting said cell with an agonist or antagonist of a Flt3 ligand. For example, the antagonist might be an antibody against a mammalian Flt3 ligand or the cell may be a hematopoietic cell, including a lymphoid cell; a placenta cell; a gonad cell; or a neural cell, including neuronal or non-neuronal cells.

DESCRIPTION OF THE INVENTION

All references cited herein are hereby incorporated in their entirety by reference.

General

The present invention provides the amino acid sequence and DNA sequences encoding various mammalian proteins that exhibit properties of binding to a tyrosine kinase receptor protein. These proteins are designated Flt3 ligands, because they were initially characterized as proteins which bind to the Flt3 protein, a protein which exhibits structural characteristics of a tyrosine kinase type of receptor.

The natural ligands are capable of mediating various biochemical responses which should lead to biological or

physiological responses in target cells. Initial studies had localized the protein to hematopoietic stem cells and primitive uncommitted progenitors. The best characterized embodiment was initially described in mouse, but human variants are also described herein. Additional sequences for proteins in other mammalian species, e.g., human, should also be available. The descriptions below are directed, for exemplary purposes, to a mouse Flt3 ligand, but are likewise applicable to related embodiments from other species.

Isolated mouse Flt3 protein was recently described as a protein which exhibits structural features of a receptor tyrosine kinase. The protein is localized in placenta, gonad, hematopoietic, and neural tissues, among others. See Matthews, *et al.* (1991) *Cell 65*:1143-1152; and Rosnet, *et al.* (1991) *Oncogene 6*:1641-1650. The Flt3 receptor mediates a biochemical response to binding of a heretofore unidentified ligand leading to signal transduction and cellular response.

In particular, the ligand has been isolated by pursuing a self-phosphorylation assay, which likely reflects cross phosphorylation of dimerized receptor molecules. The ligand has been isolated and characterized as a protein which migrates on polyacrylamide gel electrophoresis with a mobility characteristic of a protein of about 30 kD, while other physical properties are described in Table 2.

The ligand for Flt3 should be present in the mentioned tissue types, and the interaction of the ligand with receptor should be important for mediating various aspects of cellular physiology or development. The distribution of the Flt3 receptor protein in different tissues suggests that it and its ligand have functional roles outside the immune system, e.g., in developmental regulation in other cell types. See, e.g., Gilbert (1991) *Developmental Biology* (3d ed.), Sinauer Associates, Sunderland, MA; Browder, *et al.* (1991) *Developmental Biology* (3d ed.), Saunders, Philadelphia, PA.; Russo, *et al.* (1992) *Development: The Molecular Genetic*

Approach, Springer-Verlag, New York, N.Y.; and Wilkins (1993) *Genetic Analysis of Animal Development* (2d ed.) Wiley-Liss, New York, N.Y.

When highly purified native mouse Flt3 ligand was added with IL-3 to mouse Thy^{lo} $Sca\text{-}1^{+}$ lin^{-} stem cells, colony numbers were significantly increased. These conditions produced multilineage colonies, but they did not contain the abundant erythroid cells characteristically found with c-kit ligand and IL-3. A modest co-stimulatory activity was evident in the presence of Flt3 ligand and IL-6. However, Flt3 ligand alone had no stimulatory activity on these cells even when used in combination with c-kit ligand.

When sorted human fetal liver progenitor cells were used, Flt3 ligand had a similar synergistic effect in combination with GM-CSF or IL-3. In this case, the co-stimulatory effects of Flt3 ligand were observed on both low proliferative potential colony-forming cells (LPP-CFC) as well as the more primitive high proliferative potential colony-forming cells (HPP-CFC). However, in contrast to c-kit ligand, Flt3 ligand was less able to augment the growth of human fetal liver burst-forming units erythroid (BFU-E). Thus, Flt3 ligand enhances the response of stem and primitive progenitor cells to growth factors but in a manner distinct from c-kit ligand.

With respect to myeloid progenitors, Flt3 ligand has little effect alone on proliferation, but in combination with such factors as GM-CSF or IL-3, the ligand synergistically can promote the growth of both primitive and more mature myeloid precursors.

Flt3 ligand, in combination with IL-7 or IL-12, activates specific thymocyte subsets to proliferate. $CD4^{+}$, $CD8^{+}$, and $CD4^{lo}$ thymocytes are some of the subsets responding to the cytokine combinations.

Flt3 ligand was also tested on day 14 fetal thymocytes which are enriched for T cell precursors. Flt3 ligand in

combination with IL-7 or IL-12 induced significant proliferation. IL-12 also induces proliferation of fetal thymocytes in combination with c-kit ligand. These results further support a role for Flt3 ligand in T cell development.

The proliferation of early B lineage cells, e.g., a pro B cell line or bone marrow cells enriched for pro B and pre B cells, is significantly enhanced in the presence of Flt3 ligand, particularly in combination with IL-7. Early developmental stage cell populations which give rise to committed B lineage cells also proliferate in response to Flt3 ligand, particularly in combination with other stromal cell factors.

Identification of the ligand for Flt3 provides means to address some of the questions raised by these observations.

Purified Flt3 Ligand

Mouse and human Flt3 ligand amino acid sequences are shown in Table 1. These amino acid sequences, shown amino to carboxy terminus, are important in providing sequence information in the ligand allowing for distinguishing the protein from other proteins. Moreover, the peptide sequences allow preparation of peptides to generate antibodies to recognize such segments, and allow preparation of oligonucleotide probes, both of which are strategies for isolation, e.g., cloning, of genes encoding such sequences.

In particular, the MB8 isolate contains an insert of 29 amino acids which contain proteolytic processing sites which will allow the helical cytokine domain of the ligand to be cleaved from a membrane attachment. Similarities have been observed with other cytokines. See, e.g., Bosenberg, *et al.* (1992) *Cell 71*:1157-1165; Huang, *et. al.* (1992) *Molecular Biology of the Cell* 3:349-362; and Pandiella, *et al.* (1992) *J. Biol. Chem. 267*:24028-24033. This will avoid certain problems of working with, or administering, a cell bound protein, and provides insight into possible mechanisms of cellular specificity.

Table 1: Flt3 Ligand Peptide Fragment Sequences

1. FVQT(N/C/S/T)I(S)(H)LLK
2. DYPVTVAV(N)LQ(D)E(K)
3. TPD(V/A)YF(S)(H)(S)PIS(S)(N)(F)(K)
4. WIEQLK(Q)(P)(G)(S)
5. ELT(V)(H)LLK
6. ILFXLFLQ(Y)(R)
7. S(H)(S)PISSNF
8. (W)IEQLK
9. DYPVTVAVNLQ
10. DAYFSHSPISSNFKVKFREL(T)(V)
11. DYPVTVA(A)

The foregoing provides consensus sequences:

12. TPDAYFSHSPISSNFKVKFRELTVHLLK
13. WIEQLK
14. FVQTXISHLLK
15. ILFXLFAQYR
16. DYPVTVAVNLQ

Alignment with other ligands for Flt3-like receptors and other considerations suggests a mature protein N-terminal sequence:

17. TPDCYFSHSP ISSNFKVKFR ELT(V)HLLKDY PVTVAVNLQD EK

Nucleic acid sequencing of a clone provides the following sequence:

18. TPDCYFSHSP ISSNFKVKFR ELTDHLLKDY PVTVAVNLQD EKHCKALWSL FLAQRWIEQL KTVAGSKMQT LLEDVNTEIH FVTSCTFQPL PECLRFVQTN I

Five different mammalian variants have the following sequences:

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MoT118/T110  M T V L A P A W S P N S S L L L L L L L L S P C L   25
HuS86/S109   M T V L A P A W S P . T T Y L L L L L L L S S G L   24

MoT118/T110  R G T P D C Y F S H S P I S S N F K V K F R E L T   50
HuS86/S109   S G T Q D C S F Q H S P I S S D F A V K I R E L S   49

                                                          #
MoT118/T110  D H L L K D Y P V T V A V N L Q D E K H C K A L W   75
HuS86/S109   D Y L L Q D Y P V T V A S N L Q D E E L C G A L W   74

MoT118/T110  S L F L A Q R W I E Q L K T V A G S K M Q T L L E  100
HuS86/S109   R L V L A Q R W M E R L K T V A G S K M Q G L L E   99
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Table 1 (continued):

MoT118/T110	D V N T E I H F V T S C T F N P L P E C L R F V Q	125
HuS86/S109	R V N T E I H F V T K C A F Q P P P S C L R F V Q	124
MoT118/T110	T N I S H L L K D T C T Q L L A L K P C I G K A C	150
HuS86/S109	T N I S R L L Q E T S E Q L V A L K P W I T R . .	147
MoT118	Q N F S R C L E V Q C Q P G N G G P R A Q H H G A	175
MoT110	Q N F S R C L E V Q C Q P D S S T L L P P R S P I	175
HuS86	Q N F S R C L E L Q C Q P D S S T L P P P W S P R	172
HuS109	Q N F S R C L E L Q C Q P G A P R P Q S P G P A A	172
MoT118	T R L T A T A L L T V C P G L L L P L V G T S H M	200
MoT110	A L E A T E L P E P R P R Q L L L L L L L L L P L	200
HuS86	P L E A T A P T A P Q P P L L L L L L P V G L L L	197
HuS109	C G A L T W P R P H P G E D T E A H R G E S P A R	197
MoT118	F F L P Y F L S F L S S F L K M Y L Y V	220
MoT110	T L V L L A A W G L R W Q R A R R R G E L H P G .	224
HuS86	. . . L A A A W C L H W Q R T R R R T P R P G E Q	219
HuS109	G C I A W T Q R K L A R G R S L P W A P L I P S P	222
MoT110	V . P L P S H P	231
HuS86	V P P V P S P Q D L L L V E H	234
HuS109	E W R Q R Q N P A P A P F T Q L C T K P L S P	245

Bold residues are conserved with CSF's; underlined sequences are peptide sequences described above; # is the position of an insert in one clone; • indicates where divergence of sequences in variant isolates begins and the insertion point of the 29 amino acid insert found in the MB8 isolate. The MB8 isolate has a 29 amino acid insert has the sequence: DRVSLLCRLGLTLNSLQSSCL-SVLSAGIT.

The sequences shown in Table 1 are also defined in the Sequence Listing, wherein the sequences of peptides 1-18 are defined by SEQ ID NOs: 1-18, respectively. Some amino acid residues in the sequences are indicated as Xaa, where there was some uncertainty in the sequence determinations.

Other sequences shown in Table 1 are defined in the Sequence Listing as follows:

Peptide	SEQ ID NO:
HuS86/S109 (to S86 C-terminus)	19
HuS109 (C-terminal region)	20
MoT118/T110 (to T110 C-terminus)	21
MoT118 (C-terminal region)	22
MB8 Isolate	23

The nucleotide sequence for the C-terminal region of MoT118 is defined by SEQ ID NO: 24.

Table 2: Physical Properties of Mouse Flt3 Ligand

(1) SDS-Polyacrylamide gel electrophoresis: reduced migration approximately 30 Kd; seemingly a glycoprotein.

(2) Ammonium Sulfate precipitation (at 4° C): activity found in 60-85% saturated $(NH_4)_2SO_4$ pellet.

(3) Hydrophobic Interaction Chromatography [$(NH_4)_2SO_4$ gradient in 20 mM Tris, pH 7.5 on a Phenyl-5PW column]: activity eluted between 900-750 mM $(NH_4)_2SO_4$.

(4) Anion Exchange Chromatography (NaCl gradient in 20 mM Tris, pH 7.5 on Mono Q column): activity eluted between 130-250 mM NaCl.

(5) Cation Exchange Chromatography (NaCl gradient in 10 mM citrate, pH 3.0 on Mono S column): the bulk of the activity eluted between 440-540 mM NaCl.

(6) Gel Filtration (SEPHACRYL® S200 column): the activity ran with an apparent molecular weight of 70 kD.

(7) Reversed Phase HPLC (water to acetonitrile gradient in 0.1% TFA on a Poros R/H column): the activity eluted between 32-35% acetonitrile.

As used herein, the term "mouse Flt3 ligand" shall encompass, when used in a protein context, a protein having mouse amino acid sequences shown in Table 1, or a significant fragment of such a protein. It also refers to a mouse derived polypeptide which exhibits similar biological function or interacts with Flt3 ligand specific binding components. These binding components, e.g., antibodies, typically bind to a Flt3 ligand with high affinity, e.g., at least about 100 nM, usually better than about 30 nM, preferably better than about 10 nM, and more preferably at better than about 3 nM. Homologous proteins would be found in mammalian species other than mouse, e.g., rats. Non-mammalian species should also possess structurally or functionally related genes and proteins.

The term "polypeptide" as used herein includes a significant fragment or segment, and encompasses a stretch of amino acid residues of at least about 8 amino acids, generally at least 10 amino acids, more generally at least 12 amino acids, often at least 14 amino acids, more often at least 16 amino acids, typically at least 18 amino acids, more typically at least 20 amino acids, usually at least 22 amino acids, more usually at least 24 amino acids, preferably at least 26 amino acids, more preferably at least 28 amino acids, and, in particularly preferred embodiments, at least about 30 or more amino acids.

The term "binding composition" refers to molecules that bind with specificity to Flt3 ligand, e.g., in a ligand-receptor type fashion, an antibody-antigen interaction, or compounds, e.g., proteins which specifically associate with Flt3 ligand, e.g., in a natural physiologically relevant protein-protein interaction, either covalent or non-covalent. The molecule may be a polymer, or chemical reagent. No implication as to whether Flt3 ligand is either the ligand or the receptor of a ligand-receptor interaction is represented, other than the interaction exhibit similar specificity, e.g., specific affinity.

A functional analog may be a ligand with structural modifications, or may be a wholly unrelated molecule, e.g., which has a molecular shape which interacts with the appropriate ligand binding determinants. The ligands may serve as agonists or antagonists of the receptor, see, e.g., Goodman, *et al.* (eds.) (1990) *The Pharmacological Bases of Therapeutics* (8th ed.), Pergamon Press.

Substantially pure typically means that the protein is free from other contaminating proteins, nucleic acids, and other biologicals derived from the original source organism. Purity may be assayed by standard methods, and will ordinarily be at least about 40% pure, more ordinarily at least about 50% pure, generally at least about 60% pure, more generally at least about 70% pure, often at least about 75% pure, more often at least about 80% pure, typically at least about 85% pure, more typically at least about 90% pure, preferably at least about 95% pure, more preferably at least about 98% pure, and in most preferred embodiments, at least 99% pure.

Solubility of a polypeptide or fragment depends upon the environment and the polypeptide. Many parameters affect polypeptide solubility, including temperature, electrolyte environment, size and molecular characteristics of the polypeptide, and nature of the solvent. Typically, the temperature at which the polypeptide is used ranges from about 4° C to about 65° C. Usually the temperature at use is greater than about 18° C and more usually greater than about 22° C. For diagnostic purposes, the temperature will usually be about room temperature or warmer, but less than the denaturation temperature of components in the assay. For therapeutic purposes, the temperature will usually be body temperature, typically about 37° C for humans, though under certain situations the temperature may be raised or lowered *in situ* or *in vitro*.

The electrolytes will usually approximate *in situ* physiological conditions, but may be modified to higher or lower ionic strength where advantageous. The actual ions may be modified, e.g., to conform to standard buffers used in physiological or analytical contexts.

The size and structure of the polypeptide should generally be in a substantially stable state, and usually not in a denatured state. The polypeptide may be associated with other polypeptides in a quaternary structure, e.g., to confer solubility, or associated with lipids or detergents in a manner which approximates natural lipid bilayer interactions.

The solvent will usually be a biologically compatible buffer, of a type used for preservation of biological activities, and will usually approximate a physiological solvent. Usually the solvent will have a neutral pH, typically between about 5 and 10, and preferably about 7.5. On some occasions, a detergent will be added, typically a mild non-denaturing one, e.g., CHS or CHAPS, or a low enough concentration as to avoid significant disruption of structural or physiological properties of the ligand.

Solubility is reflected by sedimentation measured in Svedberg units, which are a measure of the sedimentation velocity of a molecule under particular conditions. The determination of the sedimentation velocity was classically performed in an analytical ultracentrifuge, but is typically now performed in a standard ultracentrifuge. See, Freifelder (1982) *Physical Biochemistry* (2d ed.), W.H. Freeman; and Cantor and Schimmel (1980) *Biophysical Chemistry*, parts 1-3, W.H. Freeman & Co., San Francisco. As a crude determination, a sample containing a putatively soluble polypeptide is spun in a standard full sized ultracentrifuge at about 50K rpm for about 10 minutes, and soluble molecules will remain in the supernatant. A soluble particle or polypeptide will typically be less than about 30S, more typically less than about 15S, usually less than about 10S, more usually less than about 6S,

and, in particular embodiments, preferably less than about 4S, and more preferably less than about 3S.

Two specific biological activities of the Flt3 ligand are described below. The first is a ligand-dependent activity conferred on receptor-transformed or appropriate test cells. The second is a ligand-dependent autophosphorylation of receptor. These two biological activities have been utilized to isolate an appropriate ligand.

Physical Variants

This invention also encompasses proteins or peptides having substantial amino acid sequence homology with the amino acid sequence of the Flt3 ligand. The variants include species or allelic variants.

Amino acid sequence homology, or sequence identity, is determined by optimizing residue matches, if necessary, by introducing gaps as required. This changes when considering conservative substitutions as matches. Conservative substitutions typically include substitutions within the following groups: glycine, alanine; valine, isoleucine, leucine; aspartic acid, glutamic acid; asparagine, glutamine; serine, threonine; lysine, arginine; and phenylalanine, tyrosine. Homologous amino acid sequences are typically intended to include natural allelic and interspecies variations in each respective protein sequence. Typical homologous proteins or peptides will have from 25-100% homology (if gaps can be introduced), to 50-100% homology (if conservative substitutions are included) with the amino acid sequence of the Flt3 ligand. Homology measures will be at least about 35%, generally at least 40%, more generally at least 45%, often at least 50%, more often at least 55%, typically at least 60%, more typically at least 65%, usually at least 70%, more usually at least 75%, preferably at least 80%, and more preferably at least 80%, and in particularly preferred embodiments, at least 85% or more.

See also Needleham, *et al.* (1970) *J. Mol. Biol. 48*:443-453; Sankoff, *et al.* (1983) Chapter One in *Time Warps, String Edits, and Macromolecules: The Theory and Practice of Sequence Comparison* Addison-Wesley, Reading, MA; and software packages from IntelliGenetics, Mountain View, CA; and the University of Wisconsin Genetics Computer Group, Madison, WI.

The isolated Flt3 ligand DNA can be readily modified by nucleotide substitutions, nucleotide deletions, nucleotide insertions, and inversions of nucleotide stretches. These modifications result in novel DNA sequences which encode these antigens, their derivatives, or proteins having similar physiological, immunogenic, or antigenic activity. These modified sequences can be used to produce mutant antigens or to enhance expression. Enhanced expression may involve gene amplification, increased transcription, increased translation, and other mechanisms. Such mutant Flt3 ligand derivatives include predetermined or site-specific mutations of the respective protein or its fragments. "Mutant Flt3 ligand" encompasses a polypeptide otherwise falling within the homology definition of the mouse Flt3 ligand as set forth above, but having an amino acid sequence which differs from that of Flt3 ligand as found in nature, whether by way of deletion, substitution, or insertion.

In particular, "site specific mutant Flt3 ligand" generally includes proteins having significant homology with a ligand having sequences of Table 1, and as sharing various biological activities, e.g., antigenic or immunogenic, with those sequences, and in preferred embodiments contain most of the disclosed sequences. Similar concepts apply to different Flt3 ligand proteins, particularly those found in various warm blooded animals, e.g., mammals and birds. As stated before, it is emphasized that descriptions are generally meant to encompass all Flt3 ligand proteins, not limited to the mouse embodiment specifically discussed.

Although site specific mutation sites are predetermined, mutants need not be site specific. Flt3 ligand mutagenesis can be conducted by making amino acid insertions or deletions. Substitutions, deletions, insertions, or any combinations may be generated to arrive at a final construct. Insertions include amino- or carboxy- terminal fusions. Random mutagenesis can be conducted at a target codon and the expressed mutants can then be screened for the desired activity. Methods for making substitution mutations at predetermined sites in DNA having a known sequence are well known in the art, e.g., by M13 primer mutagenesis or polymerase chain reaction (PCR) techniques. See also Sambrook, *et al.* (1989) and Ausubel, *et al.* (1987 and Supplements).

The mutations in the DNA normally should not place coding sequences out of reading frames and preferably will not create complementary regions that could hybridize to produce secondary mRNA structure such as loops or hairpins.

The present invention also provides recombinant proteins, e.g., heterologous fusion proteins using segments from these proteins. A heterologous fusion protein is a fusion of proteins or segments which are naturally not normally fused in the same manner. Thus, the fusion product of an immunoglobulin with a Flt3 ligand polypeptide is a continuous protein molecule having sequences fused in a typical peptide linkage, typically made as a single translation product and exhibiting properties derived from each source peptide. A similar concept applies to heterologous nucleic acid sequences.

In addition, new constructs may be made from combining similar functional domains from other proteins. For example, ligand-binding or other segments may be "swapped" between different new fusion polypeptides or fragments. See, e.g., Cunningham, *et al.* (1989) *Science 243*:1330-1336; and O'Dowd, *et al.* (1988) *J. Biol. Chem. 263*:15985-15992. Thus, new chimeric polypeptides exhibiting new combinations of

specificities will result from the functional linkage of ligand-binding specificities and other functional domains.

The phosphoramidite method described by Beaucage and Carruthers (1981) *Tetra. Letts.* 22:1859-1862, will produce suitable synthetic DNA fragments. A double stranded fragment will often be obtained either by synthesizing the complementary strand and annealing the strand together under appropriate conditions or by adding the complementary strand using DNA polymerase with an appropriate primer sequence, e.g., PCR techniques.

Functional Variants

The blocking of physiological response to Flt3 ligands may result from the inhibition of binding of the ligand to the Flt3 receptor, likely through competitive inhibition. Thus, *in vitro* assays of the present invention will often use isolated protein, membranes from cells expressing a recombinant membrane associated Flt3 ligand, soluble fragments comprising receptor binding segments of these ligands, or fragments attached to solid phase substrates. These assays will also allow for the diagnostic determination of the effects of either binding segment mutations and modifications, or ligand mutations and modifications, e.g., ligand analogs.

This invention also contemplates the use of competitive drug screening assays, e.g., where neutralizing antibodies to antigen or receptor fragments compete with a test compound for binding to the protein. In this manner, the antibodies can be used to detect the presence of any polypeptide which shares one or more antigenic binding sites of the ligand and can also be used to occupy binding sites on the protein that might otherwise interact with a receptor.

Additionally, neutralizing antibodies against Flt3 ligand and soluble fragments of the ligand which contain a high affinity receptor binding site, can be used to inhibit ligand

function in tissues, e.g., tissues experiencing abnormal physiology.

"Derivatives" of Flt3 ligand antigens include amino acid sequence mutants, glycosylation variants, and covalent or aggregate conjugates with other chemical moieties. Covalent derivatives can be prepared by linkage of functionalities to groups which are found in Flt3 ligand amino acid side chains or at the N- or C- termini, by means which are well known in the art. These derivatives can include, without limitation, aliphatic esters or amides of the carboxyl terminus, or of residues containing carboxyl side chains, O-acyl derivatives of hydroxyl group-containing residues, and N-acyl derivatives of the amino terminal amino acid or amino-group containing residues, e.g., lysine or arginine. Acyl groups are selected from the group of alkyl-moieties including C3 to C18 normal alkyl, thereby forming alkanoyl aroyl species. Covalent attachment to carrier proteins may be important when immunogenic moieties are haptens.

In particular, glycosylation alterations are included, e.g., made by modifying the glycosylation patterns of a polypeptide during its synthesis and processing, or in further processing steps. Particularly preferred means for accomplishing this are by exposing the polypeptide to glycosylating enzymes derived from cells which normally provide such processing, e.g., mammalian glycosylation enzymes. Deglycosylation enzymes are also contemplated. Also embraced are versions of the same primary amino acid sequence which have other minor modifications, including phosphorylated amino acid residues, e.g., phosphotyrosine, phosphoserine, or phosphothreonine.

A major group of derivatives are covalent conjugates of the Flt3 ligand or fragments thereof with other proteins or polypeptides. These derivatives can be synthesized in recombinant culture such as N- or C-terminal fusions or by the use of agents known in the art for their usefulness in cross-linking proteins through reactive side groups. Preferred

ligand derivatization sites with cross-linking agents are at free amino groups, carbohydrate moieties, and cysteine residues.

Fusion polypeptides between Flt3 ligands and other homologous or heterologous proteins are also provided. Many growth factors and cytokines are homodimeric entities, and a repeat construct may have various advantages, including lessened susceptibility to proteolytic cleavage. Moreover, many receptors require dimerization to transduce a signal, and various dimeric ligands or domain repeats can be desirable. Homologous polypeptides may be fusions between different surface markers, resulting in, e.g., a hybrid protein exhibiting receptor binding specificity. Likewise, heterologous fusions may be constructed which would exhibit a combination of properties or activities of the derivative proteins.

Typical examples are fusions of a reporter polypeptide, e.g., luciferase, with a segment or domain of a ligand, e.g., a receptor-binding segment, so that the presence or location of the fused ligand may be easily determined. See, e.g., Dull, *et al.*, U.S. Patent No. 4,859,609. Other gene fusion partners include bacterial ß-galactosidase, trpE, Protein A, ß-lactamase, alpha amylase, alcohol dehydrogenase, and yeast alpha mating factor. See, e.g., Godowski, *et al.* (1988) *Science 241*:812-816.

The phosphoramidite method described by Beaucage and Carruthers (1981) *Tetra. Letts. 22*:1859-1862, will produce suitable synthetic DNA fragments. A double stranded fragment will often be obtained either by synthesizing the complementary strand and annealing the strand together under appropriate conditions or by adding the complementary strand using DNA polymerase with an appropriate primer sequence.

Such polypeptides may also have amino acid residues which have been chemically modified by phosphorylation, sulfonation, biotinylation, or the addition or removal of other moieties, particularly those which have molecular shapes similar to phosphate groups. In some embodiments, the

modifications will be useful labeling reagents, or serve as purification targets, e.g., affinity ligands.

Fusion proteins will typically be made by either recombinant nucleic acid methods or by synthetic polypeptide methods. Techniques for nucleic acid manipulation and expression are described generally, for example, in Sambrook, *et al.* (1989) *Molecular Cloning: A Laboratory Manual* (2d ed.), Vols. 1-3, Cold Spring Harbor Laboratory. Techniques for synthesis of polypeptides are described, for example, in Merrifield (1963) *J. Amer. Chem. Soc. 85*:2149-2156; Merrifield (1986) *Science 232*: 341-347; and Atherton, *et al.* (1989) *Solid Phase Peptide Synthesis: A Practical Approach*, IRL Press, Oxford.

This invention also contemplates the use of derivatives of Flt3 ligands other than variations in amino acid sequence or glycosylation. Such derivatives may involve covalent or aggregative association with chemical moieties. These derivatives generally fall into the three classes: (1) salts, (2) side chain and terminal residue covalent modifications, and (3) adsorption complexes, for example with cell membranes. Such covalent or aggregative derivatives are useful as immunogens, as reagents in immunoassays, or in purification methods such as for affinity purification of ligands or other binding ligands. For example, a Flt3 ligand antigen can be immobilized by covalent bonding to a solid support such as cyanogen bromide-activated SEPHAROSE®, by methods which are well known in the art, or adsorbed onto polyolefin surfaces, with or without glutaraldehyde cross-linking, for use in the assay or purification of anti-Flt3 ligand antibodies or its receptor.

The Flt3 ligands can also be labeled with a detectable group, for example radioiodinated by the chloramine T procedure, covalently bound to rare earth chelates, or conjugated to another fluorescent moiety for use in diagnostic

assays. Purification of Flt3 ligand may be effected by immobilized antibodies or receptor.

A solubilized Flt3 ligand or fragment of this invention can be used as an immunogen for the production of antisera or antibodies specific for the ligand or any fragments thereof. The purified ligands can be used to screen monoclonal antibodies or ligand-binding fragments prepared by immunization with various forms of impure preparations containing the protein. In particular, the term "antibodies" also encompasses antigen binding fragments of natural antibodies. Purified Flt3 ligands can also be used as a reagent to detect antibodies generated in response to the presence of elevated levels of the ligand or cell fragments containing the ligand, both of which may be diagnostic of an abnormal or specific physiological or disease condition.

Additionally, ligand fragments may also serve as immunogens to produce the antibodies of the present invention, as described immediately below. For example, this invention contemplates antibodies raised against amino acid sequences encoded by nucleotide sequences shown in Table 1, or fragments of proteins containing them. In particular, this invention contemplates antibodies having binding affinity to or being raised against specific fragments which are predicted to lie outside of the lipid bilayer.

The present invention contemplates the isolation of additional closely related species variants. Southern and Northern blot analysis should establish that similar genetic entities exist in other mammals. It is likely that Flt3 ligands are widespread in species variants, e.g., rodents, lagomorphs, carnivores, artiodactyla, perissodactyla, and primates.

The invention also provides means to isolate a group of related antigens displaying both distinctness and similarities in structure, expression, and function. Elucidation of many of the physiological effects of the ligands will be greatly accelerated by the isolation and characterization of distinct

species variants of the ligands. In particular, the present invention provides useful probes for identifying additional homologous genetic entities in different species.

The isolated genes will allow transformation of cells lacking expression of a corresponding Flt3 ligand, e.g., either species types or cells which lack corresponding ligands and exhibit negative background activity. Expression of transformed genes will allow isolation of antigenically pure cell lines, with defined or single specie variants. This approach will allow for more sensitive detection and discrimination of the physiological effects of any Flt3 receptor proteins. Subcellular fragments, e.g., cytoplasts or membrane fragments, can be isolated and used.

Dissection of critical structural elements which effect the various differentiation functions provided by ligands is possible using standard techniques of modern molecular biology, particularly in comparing members of the related class. See, e.g., the homolog-scanning mutagenesis technique described in Cunningham, *et al.* (1989) *Science 243*:1339-1336; and approaches used in O'Dowd, *et al.* (1988) *J. Biol. Chem. 263*:15985-15992; and Lechleiter, *et al.* (1990) *EMBO J. 9*:4381-4390.

In particular, receptor binding segments can be substituted between species variants to determine what structural features are important in both receptor binding affinity and specificity, as well as signal transduction. An array of different ligand variants will be used to screen for ligands exhibiting combined properties of interaction with different receptor species variants.

Intracellular functions would probably involve segments of the receptor which are normally accessible to the cytosol. However, ligand internalization may occur under certain circumstances, and interaction between intracellular components and "extracellular" segments may occur. The specific segments of interaction of Flt3 ligand with other

intracellular components may be identified by mutagenesis or direct biochemical means, e.g., cross-linking or affinity methods. Structural analysis by crystallographic or other physical methods will also be applicable. Further investigation of the mechanism of signal transduction will include study of associated components which may be isolatable by affinity methods or by genetic means, e.g., complementation analysis of mutants.

Further study of the expression and control of Flt3 ligand will be pursued. The controlling elements associated with the ligands may exhibit differential developmental, tissue specific, or other expression patterns. Upstream or downstream genetic regions, e.g., control elements, are of interest. In particular, developmental or physiological variants, e.g., multiple alternatively processed forms of ligand have been found. See, e.g., Table 3. Thus, differential splicing of message may lead to membrane bound forms, soluble forms, and modified versions of ligand.

Structural studies of the ligands will lead to design of new ligands, particularly analogs exhibiting agonist or antagonist properties on the receptor. This can be combined with previously described screening methods to isolate ligands exhibiting desired spectra of activities.

Expression in other cell types will often result in glycosylation differences in a particular ligand. Various species variants may exhibit distinct functions based upon structural differences other than amino acid sequence. Differential modifications may be responsible for differential function, and elucidation of the effects are now made possible.

Thus, the present invention provides important reagents related to a physiological ligand-receptor interaction. Although the foregoing description has focused primarily upon the mouse Flt3 ligand, those of skill in the art will immediately recognize that the invention encompasses other ligands, e.g.,

rat and other mammalian species or allelic variants, as well as variants thereof.

Antibodies

Antibodies can be raised to various Flt3 ligands, including species or allelic variants, and fragments thereof, both in their naturally occurring forms and in their recombinant forms. Additionally, antibodies can be raised to Flt3 ligands in either their active forms or in their inactive forms. Anti-idiotypic antibodies are also contemplated.

Antibodies, including binding fragments and single chain versions, against predetermined fragments of the ligands can be raised by immunization of animals with conjugates of the fragments with immunogenic proteins. Monoclonal antibodies are prepared from cells secreting the desired antibody. These antibodies can be screened for binding to normal or defective Flt3 ligands, or screened for agonistic or antagonistic activity, e.g., mediated through the receptor. These monoclonal antibodies will usually bind with at least a K_D of about 1 mM, more usually at least about 300 µM, typically at least about 10 µM, more typically at least about 30 µM, preferably at least about 10 µM, and more preferably at least about 3 µM or better.

The antibodies, including antigen binding fragments, of this invention can have significant diagnostic or therapeutic value. They can be potent antagonists that bind to the receptor and inhibit ligand binding or inhibit the ability of a ligand to elicit a biological response. They also can be useful as non-neutralizing antibodies and can be coupled to toxins or radionuclides so that when the antibody binds to ligand, a cell expressing it, e.g., on its surface, is killed. Further, these antibodies can be conjugated to drugs or other therapeutic agents, either directly or indirectly by means of a linker, and may effect drug targeting.

The antibodies of this invention can also be useful in diagnostic applications. As capture or non-neutralizing antibodies, they can be screened for ability to bind to the ligands without inhibiting receptor binding. As neutralizing antibodies, they can be useful in competitive binding assays. They will also be useful in detecting or quantifying Flt3 ligand or its receptors.

Ligand fragments may be joined to other materials, particularly polypeptides, as fused or covalently joined polypeptides to be used as immunogens. A ligand and its fragments may be fused or covalently linked to a variety of immunogens, such as keyhole limpet hemocyanin, bovine serum albumin, tetanus toxoid, etc. See *Microbiology*, Hoeber Medical Division, Harper and Row, 1969; Landsteiner (1962) *Specificity of Serological Reactions*, Dover Publications, New York, and Williams, *et al.* (1967) *Methods in Immunology and Immunochemistry*, Vol. 1, Academic Press, New York for descriptions of methods of preparing polyclonal antisera. A typical method involves hyperimmunization of an animal with an antigen. The blood of the animal is then collected shortly after the repeated immunizations and the gamma globulin is isolated.

In some instances, it is desirable to prepare monoclonal antibodies from various mammalian hosts, such as mice, rodents, primates, humans, etc. Description of techniques for preparing such monoclonal antibodies may be found in, e.g., Stites, *et al.* (eds.) *Basic and Clinical Immunology* (4th ed.), Lange Medical Publications, Los Altos, CA, and references cited therein; Harlow and Lane (1988) *Antibodies: A Laboratory Manual*, CSH Press; Goding (1986) *Monoclonal Antibodies: Principles and Practice* (2d ed.) Academic Press, New York; and particularly in Kohler and Milstein (1975) in *Nature 256*:495-497, which discusses one method of generating monoclonal antibodies.

Summarized briefly, this method involves injecting an animal with an immunogen. The animal is then sacrificed and cells taken from its spleen, which are then fused with myeloma cells. The result is a hybrid cell or "hybridoma" that is capable of reproducing *in vitro*. The population of hybridomas is then screened to isolate individual clones, each of which secrete a single antibody species to the immunogen. In this manner, the individual antibody species obtained are the products of immortalized and cloned single B cells from the immune animal generated in response to a specific site recognized on the immunogenic substance.

Other suitable techniques involve *in vitro* exposure of lymphocytes to the antigenic polypeptides or alternatively to selection of libraries of antibodies in phage or similar vectors. See, Huse, *et al.* (1989) "Generation of a Large Combinatorial Library of the Immunoglobulin Repertoire in Phage Lambda," *Science 246*:1275-1281; and Ward, *et al.* (1989) *Nature 341*:544-546. The polypeptides and antibodies of the present invention may be used with or without modification, including chimeric or humanized antibodies. Frequently, the polypeptides and antibodies will be labeled by joining, either covalently or non-covalently, a substance which provides for a detectable signal.

A wide variety of labels and conjugation techniques are known and are reported extensively in both the scientific and patent literature. Suitable labels include radionuclides, enzymes, substrates, cofactors, inhibitors, fluorescent moieties, chemiluminescent moieties, magnetic particles, and the like. Patents, teaching the use of such labels include U.S. Patent Nos. 3,817,837; 3,850,752; 3,939,350; 3,996,345; 4,277,437; 4,275,149; and 4,366,241. Also, recombinant immunoglobulins may be produced, see Cabilly, U.S. Patent No. 4,816,567.

The antibodies of this invention can also be used for affinity chromatography in isolating the protein. Columns can be prepared where the antibodies are linked to a solid

support, e.g., particles, such as agarose, SEPHADEX®, or the like, where a cell lysate may be passed through the column, the column washed, followed by increasing concentrations of a mild denaturant, whereby the purified Flt3 ligand protein will be released.

The antibodies may also be used to screen expression libraries for particular expression products. Usually the antibodies used in such a procedure will be labeled with a moiety allowing easy detection of presence of antigen by antibody binding.

Antibodies raised against each Flt3 ligand will also be useful to raise anti-idiotypic antibodies. These will be useful in detecting or diagnosing various immunological conditions related to expression of the respective antigens.

Nucleic Acids

The described peptide sequences and the related reagents are useful in isolating a DNA clone encoding Flt3 ligand, e.g., from a natural source. Typically, it will be useful in isolating a gene from mouse, and similar procedures will be applied to isolate genes from other species, e.g., warm blooded animals, such as birds and mammals. See Table 3. Cross hybridization will allow isolation of ligand from other species. A number of different approaches should be available to successfully isolate a suitable nucleic acid clone.

The purified protein or defined peptides are useful for generating antibodies by standard methods, as described above. Synthetic peptides or purified protein can be presented to an immune system to generate monoclonal or polyclonal antibodies. See, e.g., Coligan (1991) *Current Protocols in Immunology* Wiley/Greene; and Harlow and Lane (1989) *Antibodies: A Laboratory Manual*, Cold Spring Harbor Press. Alternatively, the Flt3 receptor can be used as a specific binding reagent, and advantage can be taken of its specificity of binding, much like an antibody would be used.

For example, the specific binding composition could be used for screening of an expression library made from a cell line which expresses a Flt3 ligand. The screening can be standard staining of surface expressed ligand, or by panning. Screening of intracellular expression can also be performed by various staining or immunofluorescence procedures. The binding compositions could be used to affinity purify or sort out cells expressing the ligand.

The peptide segments can also be used to predict appropriate oligonucleotides to screen a library. The genetic code can be used to select appropriate oligonucleotides useful as probes for screening. See, e.g., Table 3. In combination with polymerase chain reaction (PCR) techniques, synthetic oligonucleotides will be useful in selecting correct clones from a library. Complementary sequences will also be used as probes or primers. Based upon identification of the likely amino terminus, the third peptide should be particularly useful, e.g., coupled with anchored vector or poly-A complementary PCR techniques or with complementary DNA of other peptides.

Table 3: Flt3 Ligand Peptide Sequences and Exemplary Predicted Oligonucleotide Probes/Primers

1. F V Q T (NCST) I (S) (H) L L K

a) TT(T/C) GTN CA(G/A) ACN AA(C/T) AT(A/C/T)
b) TT(T/C) GTN CA(G/A) ACN TG(T/C) AT(A/C/T)
c) TT(T/C) GTN CA(G/A) ACN AG(C/T) AT(A/C/T)
d) TT(T/C) GTN CA(G/A) ACN TCN AT(A/C/T)
e) TT(T/C) GTN CA(G/A) ACN ACN AT(A/C/T)

2. D Y P V T V A V (N) L Q (D) E
GA(T/C) TA(T/C) CCN GTN ACN GTN

3. T P D C Y F S H S
ACN CCN GA(T/C) TG(T/C) TA(T/C) TT(T/C)

4. W I E Q L K (Q)(P)(G)(S)
a) TGG AT(A/C/T) GA(G/A) CA(G/A) CTN AA(G/A)
b) TGG AT(A/C/T) GA(G/A) CA(G/A) TT(A/G) AA(G/A)

5. N F K V K F
AA(T/C) TT(T/C) AA(A/G) GTN AA(A/G) TT(T/C)

and sequence:

ACT CCT GAC TGT TAC TTC AGC CAC AGT CCC ATC TCC TCC AAC TTC AAA GTG AAG TTT AGA GAG TTG ACT GAC CAC CTG CTT AAA GAT

Sequences of various mammalian Flt3 ligand variants are:

mouse T110/MB8/T118

GAATTCGCGGCCGCGTCGAGCCTGGCGGGACTGAGCCCGAGACCTGCCCTCCTGTC

ACTTCCAAGAACCTGTCACAGGCATGAGGGGTCCCCGGCAGAG ATG ACA GTG CTG GCG CCA
MET Thr Val Leu Ala Pro 6

GCC TGG AGC CCA AAT TCC TCC CTG TTG CTG CTG TTG CTG CTG CTG AGT CCT
Ala Trp Ser Pro Asn Ser Ser Leu Leu Leu Leu Leu Leu Leu Ser Pro 23

TGC CTG CGG GGG ACA CCT GAC TGT TAC TTC AGC CAC AGT CCC ATC TCC TCC
Cys Leu Arg Gly Thr Pro Asp Cys Tyr Phe Ser His Ser Pro Ile Ser Ser 40

AAC TTC AAA GTG AAG TTT AGA GAG TTG ACT GAC CAC CTG CTT AAA GAT TAC
Asn Phe Lys Val Lys Phe Arg Glu Leu Thr Asp His Leu Leu Lys Asp Tyr 57

CCA GTC ACT GTG GCC GTC AAT CTT CAG GAC GAG AAG CAC TGC AAG GCC TTG
Pro Val Thr Val Ala Val Asn Leu Gln Asp Glu Lys His Cys Lys Ala Leu 74

TGG AGC CTC TTC CTA GCC CAG CGC TGG ATA GAG CAA CTG AAG ACT GTG GCA
Trp Ser Leu Phe Leu Ala Gln Arg Trp Ile Glu Gln Leu Lys Thr Val Ala 91

Table 3 (continued):

```
    GGG TCT AAG ATG CAA ACG CTT CTG GAG GAC GTC AAC ACC GAG ATA CAT TTT
    Gly Ser Lys Met Gln Thr Leu Leu Glu Asp Val Asn Thr Glu Ile His Phe  108
5
    GTC ACC TCA TGT ACC TTC CAG CCC CTA CCA GAA TGT CTG CGA TTC GTC CAG
    Val Thr Ser Cys Thr Phe Gln Pro Leu Pro Glu Cys Leu Arg Phe Val Gln  125

    ACC AAC ATC TCC CAC CTC CTG AAG GAC ACC TGC ACA CAG CTG CTT GCT CTG
10  Thr Asn Ile Ser His Leu Leu Lys Asp Thr Cys Thr Gln Leu Leu Ala Leu  142

    AAG CCC TGT ATC GGG AAG GCC TGC CAG AAT TTC TCT CGG TGC CTG GAG GTG
    Lys Pro Cys Ile Gly Lys Ala Cys Gln Asn Phe Ser Arg Cys Leu Glu Val  159

15                        T110
    CAG TGC CAG CCG Δ GAC TCC TCC ACC CTG CTG CCC CCA AGG AGT CCC ATA GCC
    Gln Cys Gln Pro Δ Asp Ser Ser Thr Leu Leu Pro Pro Arg Ser Pro Ile Ala 176

    CTA GAA GCC ACG GAG CTC CCA GAG CCT CGG CCC AGG CAG CTG TTG CTC CTG
20  Leu Glu Ala Thr Glu Leu Pro Glu Pro Arg Pro Arg Gln Leu Leu Leu Leu  193

    CTG CTG CTG CTG CTG CCT CTC ACA CTG GTG CTG CTG GCA GCC GCC TGG GGC
    Leu Leu Leu Leu Leu Pro Leu Thr Leu Val Leu Leu Ala Ala Ala Trp Gly  210

25  CTT CGC TGG CAA AGG GCA AGA AGG AGG GGG GAG CTC CAC CCT GGG GTG CCC
    Leu Arg Trp Gln Arg Ala Arg Arg Arg Gly Glu Leu His Pro Gly Val Pro  227

    CTC CCC TCC CAT CCC TAGGATGCGAGCCTTGTGCATCGTTGACTCAGCCAGGGTCTTATCTC
    Leu Pro Ser His Pro                                                 232
30
    GAGTTGGGAACCAAAACAAGGAACAAGCTAGGCAAGTGCTGTGCTGAGTTACATCCCCAGCCCAGAG
    GACACACTGTCTGGGTATGGCGATGGACACTGTAATTCCAGTGCTTCTGGATTGGACATGCTGAAAC
    TGGATACTGACTTTAAGAAAAACAGAAAGGAAGAACCCCCC

35  MB8 insert (29 amino acids)
      [TGC CAG CCG] GAT AGG GTC TCA TTA TTA TGC AGG CTA GGC CTG ACC CTG
                ...  Asp Arg Val Ser Leu Leu Cys Arg Leu Gly Leu Thr Leu   Δ13

    AAC TCA AAG CAA TCC TCC TGC CTC AGT GTC CTG AGT GCT GGG ATT ACA [GAC TCC]
40  Asn Ser Lys Gln Ser Ser Cys Leu Ser Val Leu Ser Ala Gly Ile Thr ...   Δ29

                  or T118
              ... GGT AAC GGT GGC CCC AGA GCC CAG CAC CAT GGT GCC ACC
              ... Gly Asn Gly Gly Pro Arg Ala Gln His His Gly Ala Thr 176
45
    AGG CTC ACA GCC ACA GCC TTG CTA ACT GTG TGT CCA GGG CTT CTG CTC CCA
    Arg Leu Thr Ala Thr Ala Leu Leu Thr Val Cys Pro Gly Leu Leu Leu Pro  193

    CTA GTT GGC ACT TCA CAC ATG TTC TTT CTC CCT TAT TTT CTC TCT TTT CTT
50  Leu Val Gly Thr Ser His Met Phe Phe Leu Pro Tyr Phe Leu Ser Phe Leu  210

    TCT TCT TTT TTA AAG ATG TAT CTT TAT GTG TGAGTGTTTTACCTACATGCCTGTAAG
    Ser Ser Phe Leu Lys Met Tyr Leu Tyr Val                             220
```

Table 3 (continued)

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TGCACTGAATGTGTGTCTGGTGCCTGCAGAGGCCAGAAGAGGGCACCAGATCCCCTGAAACTGGAGT
CTCTNNGCTCCGTGTGAACCACCACGTGGTGCTGGGACCCAGGTCCAATGCAAGAGCACCCAGGGTT
CTTACCTGCTGA
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human S86/S109

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                    GAAAGGGCTGTCACCCGGCTTGGCCCCTTCCACACCCAACTGGGGCAAGCC

TGACCCGGCGACAGGAGGCATGAGGGGCCCCCGGCCGAA ATG ACA GTG CTG GCG CCA GCC
                                          MET Thr Val Leu Ala Pro Ala    7

TGG AGC CCA ACA ACC TAT CTC CTC CTG CTG CTG CTG CTG AGC TCG GGA CTC
Trp Ser Pro Thr Thr Tyr Leu Leu Leu Leu Leu Leu Leu Ser Ser Gly Leu   24

AGT GGG ACC CAG GAC TGC TCC TTC CAA CAC AGC CCC ATC TCC TCC GAC TTC
Met Gly Thr Gln Asp Cys Ser Phe Gln His Ser Pro Ile Ser Ser Asp Phe   41

GCT GTC AAA ATC CGT GAG CTG TCT GAC TAC CTG CTT CAA GAT TAC CCA GTC
Ala Val Lys Ile Arg Glu Leu Ser Asp Tyr Leu Leu Gln Asp Tyr Pro Val   58
                                  #
ACC GTG GCC TCC AAC CTG CAG GAC GAG GAG CTC TGC GGG GCG CTC TGG CGG
Thr Val Ala Ser Asn Leu Gln Asp Glu Glu Leu Cys Gly Ala Leu Trp Arg   75

CTG GTC CTG GCA CAG CGC TGG ATG GAG CGG CTC AAG ACT GTC GCT GGG TCC
Leu Val Leu Ala Gln Arg Trp Met Glu Arg Leu Lys Thr Val Ala Gly Ser   92

AAG ATG CAA GGC TTG CTG GAG CGC GTG AAC ACG GAG ATA CAC TTT GTC ACC
Lys Met Gln Gly Leu Leu Glu Arg Val Asn Thr Glu Ile His Phe Val Thr  109

AAA TGT GCC TTT CAG CCC CCC CCC AGC TGT CTT CGC TTC GTC CAG ACC AAC
Lys Cys Ala Phe Gln Pro Pro Pro Ser Cys Leu Arg Phe Val Gln Thr Asn  126

ATC TCC CGC CTC CTG CAG GAG ACC TCC GAG CAG CTG GTG GCG CTG AAG CCC
Ile Ser Arg Leu Leu Gln Glu Thr Ser Glu Gln Leu Val Ala Leu Lys Pro  143

TGG ATC ACT CGC CAG AAC TTC TCC CGG TGC CTG GAG CTG CAG TGT CAG CCC
Trp Ile Thr Arg Gln Asn Phe Ser Arg Cys Leu Glu Leu Gln Cys Gln Pro  160

S86
GAC TCC TCA ACC CTG CCA CCC CCA TGG AGT CCC CGG CCC CTG GAG GCC ACA
Asp Ser Ser Thr Leu Pro Pro Pro Trp Ser Pro Arg Pro Leu Glu Ala Thr  177

GCC CCG ACA GCC CCG CAG CCC CCT CTG CTC CTC CTA CTG CTG CTG CCC GTG
Ala Pro Thr Ala Pro Gln Pro Pro Leu Leu Leu Leu Leu Leu Leu Pro Val  194

GGC CTC CTG CTG CTG GCC GCT GCC TGG TGC CTG CAC TGG CAG AGG ACG CGG
Gly Leu Leu Leu Leu Ala Ala Ala Trp Cys Leu His Trp Gln Arg Thr Arg  211

CGG AGG ACA CCC CGC CCT GGG GAG CAG GTG CCC CCC GTC CCC AGT CCC CAG
Arg Arg Thr Pro Arg Pro Gly Glu Gln Val Pro Pro Val Pro Ser Pro Gln  228

GAC CTG CTG CTT GTG GAG CAC TGACCTGGCCAAGGCCTCATCCTGGGGAGGATACGTAGG
Asp Leu Leu Leu Val Glu His                                         235

CACACAGAGGGGAGTCACCAGCC
```

Table 3 (continued):

```
or  S109
GGT GCC CCC CGT CCC CAG TCC CCA GGA CCT GCT GCT TGT GGA GCA CTG ACC
Gly Ala Pro Arg Pro Gln Ser Pro Gly Pro Ala Ala Cys Gly Ala Leu Thr  177

TGG CCA AGG CCT CAT CCT GGG GAG GAT ACT GAG GCA CAC AGA GGG GAG TCA
Trp Pro Arg Pro His Pro Gly Glu Asp Thr Glu Ala His Arg Gly Glu Ser  194

CCA GCC AGA GGA TGC ATA GCC TGG ACA CAG AGG AAG TTG GCT AGA GGC CGG
Pro Ala Arg Gly Cys Ile Ala Trp Thr Gln Arg Lys Leu Ala Arg Gly Arg  211

TCC CTT CCT TGG GCC CCT CTC ATT CCC TCC CCA GAA TGG AGG CAA CGC CAG
Ser Leu Pro Trp Ala Pro Leu Ile Pro Ser Pro Glu Trp Arg Gln Arg Gln  228

AAT CCA GCA CCG GCC CCA TTT ACC CAA CTC TGT ACA AAG CCC TTG TCC CCA
Asn Pro Ala Pro Ala Pro Phe Thr Gln Leu Cys Thr Lys Pro Leu Ser Pro  245

TGAAATTGTATATAAATCATCCTTTTCTACCAAAAAAAAAAAAAAA
```

Of course the complementary sequences are also useful.

The sequences shown in Table 3 are also defined in the Sequence Listing as follows:

Peptide	SEQ ID NO:
1. a)	25
b)	26
c)	27
d)	28
e)	29
2.	30
3.	31
4. a)	32
b)	33
5.	34
Nucleotide Sequence	35
MoT110/T118	21
MB8	23

Peptide	SEQ ID NO:
T118	
Nucleotide Sequence	24
Amino Acid Sequence	22
HuS86/S109	19
S109	20

An isolated nucleic acid encoding an amino terminal segment has been isolated and sequenced and provides the following sequence: ACT CCT GAC TGT TAC TTC AGC CAC AGT CCC ATC TCC TCC AAC TTC AAA GTG AAG TTT AGA GAG TTG ACT GAC CAC CTG CTT AAA GAT. This may be used as a probe to isolate a longer or full length clone and will lead to isolation of other species or allelic variants or other closely related genes. See Table 3 and Table 5, below.

This invention contemplates use of isolated DNA or fragments to encode a biologically active corresponding Flt3 ligand polypeptide. In addition, this invention covers isolated or recombinant DNA which encodes a biologically active protein or polypeptide which is capable of hybridizing under appropriate conditions with the DNA sequences described herein. Said biologically active protein or polypeptide can be an intact ligand, or fragment, and have an amino acid sequence as disclosed in Table 1. Further, this invention covers the use of isolated or recombinant DNA, or fragments thereof, which encode proteins which are homologous to a Flt3 ligand or which was isolated using cDNA encoding a Flt3 ligand as a probe. The isolated DNA can have the respective regulatory sequences in the 5' and 3' flanks, e.g., promoters, enhancers, poly-A addition signals, and others.

An "isolated" nucleic acid is a nucleic acid, e.g., an RNA, DNA, or a mixed polymer, which is substantially separated from other components which naturally accompany a native

sequence, e.g., ribosomes, polymerases, and flanking genomic sequences from the originating species. The term embraces a nucleic acid sequence which has been removed from its naturally occurring environment, and includes recombinant or cloned DNA isolates and chemically synthesized analogs or analogs biologically synthesized by heterologous systems. A substantially pure molecule includes isolated forms of the molecule.

An isolated nucleic acid will generally be a homogeneous composition of molecules, but will, in some embodiments, contain minor heterogeneity. This heterogeneity is typically found at the polymer ends or portions not critical to a desired biological function or activity.

A "recombinant" nucleic acid is defined either by its method of production or its structure. In reference to its method of production, e.g., a product made by a process, the process is use of recombinant nucleic acid techniques, e.g., involving human intervention in the nucleotide sequence, typically selection or production. Alternatively, it can be a nucleic acid made by generating a sequence comprising fusion of two fragments which are not naturally contiguous to each other, but is meant to exclude products of nature, e.g., naturally occurring mutants. Thus, for example, products made by transforming cells with any unnaturally occurring vector is encompassed, as are nucleic acids comprising sequence derived using any synthetic oligonucleotide process. Such is often done to replace a codon with a redundant codon encoding the same or a conservative amino acid, while typically introducing or removing a sequence recognition site.

Alternatively, it is performed to join together nucleic acid segments of desired functions to generate a single genetic entity comprising a desired combination of functions not found in the commonly available natural forms. Restriction enzyme recognition sites are often the target of such artificial manipulations, but other site specific targets, e.g., promoters,

DNA replication sites, regulation sequences, control sequences, or other useful features may be incorporated by design. A similar concept is intended for a recombinant, e.g., fusion, polypeptide. Specifically included are synthetic nucleic acids which, by genetic code redundancy, encode polypeptides similar to fragments of these antigens, and fusions of sequences from various different species variants.

A significant "fragment" in a nucleic acid context is a contiguous segment of at least about 17 nucleotides, generally at least 20 nucleotides, more generally at least 23 nucleotides, ordinarily at least 26 nucleotides, more ordinarily at least 29 nucleotides, often at least 32 nucleotides, more often at least 35 nucleotides, typically at least 38 nucleotides, more typically at least 41 nucleotides, usually at least 44 nucleotides, more usually at least 47 nucleotides, preferably at least 50 nucleotides, more preferably at least 53 nucleotides, and in particularly preferred embodiments will be at least 56 or more nucleotides.

A DNA which codes for a Flt3 ligand protein will be particularly useful to identify genes, mRNA, and cDNA species which code for related or homologous ligands, as well as DNAs which code for homologous proteins from different species. There are likely homologues in other species, including primates. Various Flt3 ligand proteins should be homologous and are encompassed herein. However, even proteins that have a more distant evolutionary relationship to the ligand can readily be isolated under appropriate conditions using these sequences if they are sufficiently homologous. Primate Flt3 ligand proteins are of particular interest.

This invention further covers recombinant DNA molecules and fragments having a DNA sequence identical to or highly homologous to the isolated DNAs set forth herein. In particular, the sequences will often be operably linked to DNA segments which control transcription, translation, and DNA replication. Alternatively, recombinant clones derived from

the genomic sequences, e.g., containing introns, will be useful for transgenic studies, including, e.g., transgenic cells and organisms, and for gene therapy. See, e.g., Goodnow (1992) "Transgenic Animals" in Roitt (ed.) *Encyclopedia of Immunology* Academic Press, San Diego, pp. 1502-1504; Travis (1992) *Science 256*:1392-1394; Kuhn, *et al.* (1991) *Science 254*:707-710; Capecchi (1989) *Science 244*:1288; Robertson (1987)(ed.) *Teratocarcinomas and Embryonic Stem Cells: A Practical Approach* IRL Press, Oxford; and Rosenberg (1992) *J. Clinical Oncology 10*:180-199.

Homologous nucleic acid sequences, when compared, exhibit significant similarity. The standards for homology in nucleic acids are either measures for homology generally used in the art by sequence comparison or based upon hybridization conditions. The hybridization conditions are described in greater detail below.

Substantial homology in the nucleic acid sequence comparison context means either that the segments, or their complementary strands, when compared, are identical when optimally aligned, with appropriate nucleotide insertions or deletions, in at least about 50% of the nucleotides, generally at least 56%, more generally at least 59%, ordinarily at least 62%, more ordinarily at least 65%, often at least 68%, more often at least 71%, typically at least 74%, more typically at least 77%, usually at least 80%, more usually at least about 85%, preferably at least about 90%, more preferably at least about 95 to 98% or more, and in particular embodiments, as high at about 99% or more of the nucleotides.

Alternatively, substantial homology exists when the segments will hybridize under selective hybridization conditions, to a strand, or its complement, typically using a sequence derived from Table 2. Typically, selective hybridization will occur when there is at least about 55% homology over a stretch of at least about 30 nucleotides, preferably at least about 65% over a stretch of at least about

25 nucleotides, more preferably at least about 75%, and most preferably at least about 90% over about 20 nucleotides. See, Kanehisa (1984) *Nuc. Acids Res. 12*:203-213.

The length of homology comparison, as described, may be over longer stretches, and in certain embodiments will be over a stretch of at least about 17 nucleotides, usually at least about 20 nucleotides, more usually at least about 24 nucleotides, typically at least about 28 nucleotides, more typically at least about 40 nucleotides, preferably at least about 50 nucleotides, and more preferably at least about 75 to 100 or more nucleotides.

Stringent conditions, in referring to homology in the hybridization context, will be stringent combined conditions of salt, temperature, organic solvents, and other parameters, typically those controlled in hybridization reactions. Stringent temperature conditions will usually include temperatures in excess of about 30° C, more usually in excess of about 37° C, typically in excess of about 45° C, more typically in excess of about 55° C, preferably in excess of about 65° C, and more preferably in excess of about 70° C. Stringent salt conditions will ordinarily be less than about 1000 mM, usually less than about 500 mM, more usually less than about 400 mM, typically less than about 300 mM, preferably less than about 200 mM, and more preferably less than about 150 mM. However, the combination of parameters is much more important than the measure of any single parameter. See, e.g., Wetmur and Davidson (1968) *J. Mol. Biol. 31*:349-370.

Flt3 ligand from other mammalian species can be cloned and isolated by cross-species hybridization of closely related species. See, e.g., below. Homology may be relatively low between distantly related species, and thus hybridization of relatively closely related species is advisable. Alternatively, preparation of an antibody preparation which exhibits less species specificity may be useful in expression cloning approaches.

Making Flt3 Ligand: Mimetics

DNA which encodes the Flt3 ligand or fragments thereof can be obtained by chemical synthesis, screening cDNA libraries, or by screening genomic libraries prepared from a wide variety of cell lines or tissue samples.

This DNA can be expressed in a wide variety of host cells for the synthesis of a full-length ligand or fragments which can in turn, for example, be used to generate polyclonal or monoclonal antibodies; for binding studies; for construction and expression of modified molecules; and for structure/function studies. Each antigen or its fragments can be expressed in host cells that are transformed or transfected with appropriate expression vectors. These molecules can be substantially purified to be free of protein or cellular contaminants, other than those derived from the recombinant host, and therefore are particularly useful in pharmaceutical compositions when combined with a pharmaceutically acceptable carrier and/or diluent. The antigen, or portions thereof, may be expressed as fusions with other proteins.

Expression vectors are typically self-replicating DNA or RNA constructs containing the desired antigen gene or its fragments, usually operably linked to suitable genetic control elements that are recognized in a suitable host cell. These control elements are capable of effecting expression within a suitable host. The specific type of control elements necessary to effect expression will depend upon the eventual host cell used. Generally, the genetic control elements can include a prokaryotic promoter system or a eukaryotic promoter expression control system, and typically include a transcriptional promoter, an optional operator to control the onset of transcription, transcription enhancers to elevate the level of mRNA expression, a sequence that encodes a suitable ribosome binding site, and sequences that terminate transcription and translation. Expression vectors also usually

contain an origin of replication that allows the vector to replicate independently of the host cell.

The vectors of this invention contain DNA which encodes a Flt3 ligand, or a fragment thereof, typically encoding a biologically active polypeptide. The DNA can be under the control of a viral promoter and can encode a selection marker. This invention further contemplates use of such expression vectors which are capable of expressing eukaryotic cDNA coding for a Flt3 ligand in a prokaryotic or eukaryotic host, where the vector is compatible with the host and where the eukaryotic cDNA coding for the ligand is inserted into the vector such that growth of the host containing the vector expresses the cDNA in question. Usually, expression vectors are designed for stable replication in their host cells or for amplification to greatly increase the total number of copies of the desirable gene per cell. It is not always necessary to require that an expression vector replicate in a host cell, e.g., it is possible to effect transient expression of the ligand or its fragments in various hosts using vectors that do not contain a replication origin that is recognized by the host cell. It is also possible to use vectors that cause integration of a Flt3 ligand gene or its fragments into the host DNA by recombination, or to integrate a promoter which controls expression of an endogenous gene.

Vectors, as used herein, comprise plasmids, viruses, bacteriophage, integratable DNA fragments, and other vehicles which enable the integration of DNA fragments into the genome of the host. Expression vectors are specialized vectors which contain genetic control elements that effect expression of operably linked genes. Plasmids are the most commonly used form of vector but all other forms of vectors which serve an equivalent function and which are, or become, known in the art are suitable for use herein. See, e.g., Pouwels, *et al.* (1985 and Supplements) *Cloning Vectors: A Laboratory Manual*, Elsevier, N.Y., and Rodriquez, *et al.* (1988)(eds.)

Vectors: A Survey of Molecular Cloning Vectors and Their Uses, Buttersworth, Boston, MA.

Transformed cells include cells, preferably mammalian, that have been transformed or transfected with Flt3 ligand gene containing vectors constructed using recombinant DNA techniques. Transformed host cells usually express the ligand or its fragments, but for purposes of cloning, amplifying, and manipulating its DNA, do not need to express the protein. This invention further contemplates culturing transformed cells in a nutrient medium, thus permitting the protein to accumulate in the culture. The protein can be recovered, either from the culture or from the culture medium.

For purposes of this invention, DNA sequences are operably linked when they are functionally related to each other. For example, DNA for a presequence or secretory leader is operably linked to a polypeptide if it is expressed as a preprotein or participates in directing the polypeptide to the cell membrane or in secretion of the polypeptide. A promoter is operably linked to a coding sequence if it controls the transcription of the polypeptide; a ribosome binding site is operably linked to a coding sequence if it is positioned to permit translation. Usually, operably linked means contiguous and in reading frame, however, certain genetic elements such as repressor genes are not contiguously linked but still bind to operator sequences that in turn control expression.

Suitable host cells include prokaryotes, lower eukaryotes, and higher eukaryotes. Prokaryotes include both gram negative and gram positive organisms, e.g., *E. coli* and *B. subtilis*. Lower eukaryotes include yeasts, e.g., *S. cerevisiae* and *Pichia*, and species of the genus *Dictyostelium*. Higher eukaryotes include established tissue culture cell lines from animal cells, both of non-mammalian origin, e.g., insect cells, and birds, and of mammalian origin, e.g., human, primates, and rodents.

Prokaryotic host-vector systems include a wide variety of vectors for many different species. As used herein, *E. coli* and its vectors will be used generically to include equivalent vectors used in other prokaryotes. A representative vector for amplifying DNA is pBR322 or many of its derivatives. Vectors that can be used to express the Flt3 ligands or its fragments include, but are not limited to, such vectors as those containing the lac promoter (pUC-series); trp promoter (pBR322-trp); Ipp promoter (the pIN-series); lambda-pP or pR promoters (pOTS); or hybrid promoters such as ptac (pDR540). See Brosius, *et al.* (1988) "Expression Vectors Employing Lambda-, trp-, lac-, and Ipp-derived Promoters", in Rodriguez and Denhardt (eds.) *Vectors: A Survey of Molecular Cloning Vectors and Their Uses*, Buttersworth, Boston, Chapter 10, pp. 205-236.

Lower eukaryotes, e.g., yeasts and Dictyostelium, may be transformed with Flt3 ligand sequence containing vectors. For purposes of this invention, the most common lower eukaryotic host is the baker's yeast, Saccharomyces cerevisiae. It will be used to generically represent lower eukaryotes although a number of other strains and species are also available. Yeast vectors typically consist of a replication origin (unless of the integrating type), a selection gene, a promoter, DNA encoding the desired protein or its fragments, and sequences for translation termination, polyadenylation, and transcription termination.

Suitable expression vectors for yeast include such constitutive promoters as 3-phosphoglycerate kinase and various other glycolytic enzyme gene promoters or such inducible promoters as the alcohol dehydrogenase 2 promoter or metallothionine promoter. Suitable vectors include derivatives of the following types: self-replicating low copy number (such as the YRp-series), self-replicating high copy number (such as the YEp-series); integrating types (such as the YIp-series), or mini-chromosomes (such as the YCp-series).

Higher eukaryotic tissue culture cells are the preferred host cells for expression of the functionally active Flt3 ligand protein. In principle, any higher eukaryotic tissue culture cell line is workable, e.g., insect baculovirus expression systems, whether from an invertebrate or vertebrate source. However, mammalian cells are preferred, in that the processing, both cotranslationally and posttranslationally. Transformation or transfection and propagation of such cells has become a routine procedure.

Examples of useful cell lines include HeLa cells, Chinese hamster ovary (CHO) cell lines, baby rat kidney (BRK) cell lines, insect cell lines, bird cell lines, and monkey (COS) cell lines. Expression vectors for such cell lines usually include an origin of replication, a promoter, a translation initiation site, RNA splice sites (if genomic DNA is used), a polyadenylation site, and a transcription termination site. These vectors also usually contain a selection gene or amplification gene.

Suitable expression vectors may be plasmids, viruses, or retroviruses carrying promoters derived, e.g., from such sources as from adenovirus, SV40, parvoviruses, vaccinia virus, or cytomegalovirus. Representative examples of suitable expression vectors include pCDNA1; pCD, see Okayama, *et al.* (1985) *Mol. Cell Biol. 5*:1136-1142; pMC1neo Poly-A, see Thomas, *et al.* (1987) *Cell 51*:503-512; and a baculovirus vector such as pAC 373 or pAC 610.

It will often be desired to express a Flt3 ligand polypeptide in a system which provides a specific or defined glycosylation pattern. In this case, the usual pattern will be that provided naturally by the expression system. However, the pattern will be modifiable by exposing the polypeptide, e.g., an unglycosylated form, to appropriate glycosylating proteins introduced into a heterologous expression system. For example, the Flt3 ligand gene may be co-transformed with one or more genes encoding mammalian or other glycosylating enzymes. Using this approach, certain mammalian

glycosylation patterns will be achievable or approximated in prokaryote or other cells.

The Flt3 ligand, or a fragment thereof, may be engineered to be phosphatidyl inositol (PI) linked to a cell membrane, but can be removed from membranes by treatment with a phosphatidyl inositol cleaving enzyme, e.g., phosphatidyl inositol phospholipase-C. This releases the antigen in a biologically active form, and allows purification by standard procedures of protein chemistry. See, e.g., Low (1989) *Biochim. Biophys. Acta* 988:427-454; Tse, *et al.* (1985) *Science 230*:1003-1008; and Brunner, *et al.* (1991) *J. Cell Biol. 114*:1275-1283.

Now that the Flt3 ligand has been characterized, fragments or derivatives thereof can be prepared by conventional processes for synthesizing peptides. These include processes such as are described in Stewart and Young (1984) *Solid Phase Peptide Synthesis*, Pierce Chemical Co., Rockford, IL; Bodanszky and Bodanszky (1984) *The Practice of Peptide Synthesis*, Springer-Verlag, New York; and Bodanszky (1984) *The Principles of Peptide Synthesis*, Springer-Verlag, New York.

For example, an azide process, an acid chloride process, an acid anhydride process, a mixed anhydride process, an active ester process (for example, p-nitrophenyl ester, N-hydroxysuccinimide ester, or cyanomethyl ester), a carbodiimidazole process, an oxidative-reductive process, or a dicyclohexylcarbodiimide (DCCD)/additive process can be used. Solid phase and solution phase syntheses are both applicable to the foregoing processes.

The Flt3 ligand, fragments, or derivatives are suitably prepared in accordance with the above processes as typically employed in peptide synthesis, generally either by a so-called stepwise process which comprises condensing an amino acid to the terminal amino acid, one by one in sequence, or by coupling peptide fragments to the terminal amino acid. Amino

groups that are not being used in the coupling reaction are typically protected to prevent coupling at an incorrect location.

If a solid phase synthesis is adopted, the C-terminal amino acid is bound to an insoluble carrier or support through its carboxyl group. The insoluble carrier is not particularly limited as long as it has a binding capability to a reactive carboxyl group. Examples of such insoluble carriers include halomethyl resins, such as chloromethyl resin or bromomethyl resin, hydroxymethyl resins, phenol resins, tert-alkyloxycarbonyl-hydrazidated resins, and the like.

An amino group-protected amino acid is bound in sequence through condensation of its activated carboxyl group and the reactive amino group of the previously formed peptide or chain, to synthesize the peptide step by step. After synthesizing the complete sequence, the peptide is split off from the insoluble carrier to produce the peptide. This solid-phase approach is generally described by Merrifield, *et al.* (1963) in *J. Am. Chem. Soc. 85*:2149-2156.

The prepared ligand and fragments thereof can be isolated and purified from the reaction mixture by means of peptide separation, for example, by extraction, precipitation, electrophoresis and various forms of chromatography, and the like. The Flt3 ligands of this invention can be obtained in varying degrees of purity depending upon its desired use. Purification can be accomplished by use of the protein purification techniques disclosed herein or by the use of the antibodies herein described in immunoabsorbant affinity chromatography.

This immunoabsorbant affinity chromatography is carried out by first linking the antibodies to a solid support and then contacting the linked antibodies with solubilized lysates of appropriate source cells, lysates of other cells expressing the ligand, or lysates or supernatants of cells producing the Flt3 ligand as a result of DNA techniques, see below.

Uses

The present invention provides reagents which will find use in diagnostic applications as described elsewhere herein, e.g., in the general description for developmental abnormalities, or below in the description of kits for diagnosis.

This invention also provides reagents with significant therapeutic value. The Flt3 ligand (naturally occurring or recombinant), fragments thereof and antibodies thereto, along with compounds identified as having binding affinity to Flt3 ligand, should be useful in the treatment of conditions associated with abnormal physiology or development, including abnormal proliferation, e.g., cancerous conditions, or degenerative conditions. In particular, modulation of development of lymphoid cells is likely, but the wider tissue distribution on non-lymphoid tissues, e.g., gonads and neural cells, suggests that development of those tissues will be similarly responsive.

Abnormal proliferation, regeneration, degeneration, and atrophy may be modulated by appropriate therapeutic treatment using the compositions provided herein. For example, a disease or disorder associated with abnormal expression or abnormal signaling by a Flt3 ligand should be a likely target for an agonist or antagonist of the ligand. The ligand likely plays a role in regulation or development of hematopoietic cells, e.g., lymphoid cells, which affect immunological responses, e.g., autoimmune disorders.

Other abnormal developmental conditions are known in each of the cell types shown to possess Flt3 receptor mRNA by Northern blot analysis. See Berkow (ed.) *The Merck Manual of Diagnosis and Therapy*, Merck & Co., Rahway, N.J.; and Thorn, *et al. Harrison's Principles of Internal Medicine*. McGraw-Hill, N.Y. For example, neural and brain abnormalities exist in, e.g., cerebrovascular disease, CNS neoplasms, demyelinating diseases, and muscular dystrophies. Liver disorders, kidney

disorders, cardiopulmonary disorders, and other problems often cause medical symptoms. These problems may be susceptible to prevention or treatment using compositions provided herein.

Recombinant Flt3 or Flt3 ligand antibodies can be purified and then administered to a patient. These reagents can be combined for therapeutic use with additional active or inert ingredients, e.g., in conventional pharmaceutically acceptable carriers or diluents, e.g., immunogenic adjuvants, along with physiologically innocuous stabilizers and excipients. These combinations can be sterile filtered and placed into dosage forms as by lyophilization in dosage vials or storage in stabilized aqueous preparations. This invention also contemplates use of antibodies or binding fragments thereof, including forms which are not complement binding.

Drug screening using Flt3 receptor or fragments thereof can be performed to identify compounds having binding affinity to Flt3 ligand, including isolation of associated components. Subsequent biological assays can then be utilized to determine if the compound has intrinsic stimulating activity and is therefore a blocker or antagonist in that it blocks the activity of the ligand. Likewise, a compound having intrinsic stimulating activity can activate the receptor and is thus an agonist in that it simulates the activity of Flt3 ligand. This invention further contemplates the therapeutic use of antibodies to Flt3 ligand as antagonists. This approach should be particularly useful with other Flt3 ligand species variants.

The quantities of reagents necessary for effective therapy will depend upon many different factors, including means of administration, target site, physiological state of the patient, and other medicaments administered. Thus, treatment dosages should be titrated to optimize safety and efficacy. Typically, dosages used *in vitro* may provide useful guidance in the amounts useful for *in situ* administration of these reagents. Animal testing of effective doses for

treatment of particular disorders will provide further predictive indication of human dosage.

Various considerations are described, e.g., in Gilman, *et al.* (eds.) (1990) *The Pharmacological Bases of Therapeutics*, 8th Ed., Pergamon Press; and *Remington's Pharmaceutical Sciences*, 17th ed. (1990), Mack Publishing Co., Easton, Penn. Methods for administration are discussed therein and below, e.g., for oral, intravenous, intraperitoneal, or intramuscular administration, transdermal diffusion, and others.

Pharmaceutically acceptable carriers will include water, saline, buffers, and other compounds described, e.g., in the *Merck Index*, Merck & Co., Rahway, New Jersey. Dosage ranges would ordinarily be expected to be in amounts lower than 1 mM concentrations, typically less than about 10 µM concentrations, usually less than about 100 nM, preferably less than about 10 pM (picomolar), and most preferably less than about 1 fM (femtomolar), with an appropriate carrier. Slow release formulations, or a slow release apparatus will often be utilized for continuous administration.

Flt3 ligand, fragments thereof, and antibodies to it or its fragments, antagonists, and agonists, may be administered directly to the host to be treated or, depending on the size of the compounds, it may be desirable to conjugate them to carrier proteins such as ovalbumin or serum albumin prior to their administration. Therapeutic formulations may be administered in any conventional dosage formulation.

While it is possible for the active ingredient to be administered alone, it is preferable to present it as a pharmaceutical formulation. Formulations typically comprise at least one active ingredient, as defined above, together with one or more acceptable carriers thereof. Each carrier should be both pharmaceutically and physiologically acceptable in the sense of being compatible with the other ingredients and not injurious to the patient. Formulations include those suitable

for oral, rectal, nasal, or parenteral (including subcutaneous, intramuscular, intravenous and intradermal) administration.

The formulations may conveniently be presented in unit dosage form and may be prepared by any methods well known in the art of pharmacy. See, e.g., Gilman, *et al.* (eds.) (1990) *The Pharmacological Bases of Therapeutics*, *supra*; and *Remington's Pharmaceutical Sciences*, *supra*; Avis, *et al.* (eds.) (1993) *Pharmaceutical Dosage Forms: Parenteral Medications* Dekker, New York; Lieberman, *et al.* (eds.) (1990) *Pharmaceutical Dosgae Forms: Tablets*, Dekker, New York; and Lieberman, *et al.* (eds.) (1990) *Pharmaceutical Dosage Forms: Disperse Systems*, Dekker, New York. The therapy of this invention may be combined with or used in association with other chemotherapeutic or chemopreventive agents.

Both the naturally occurring and the recombinant form of the Flt3 ligands of this invention are particularly useful in kits and assay methods which are capable of screening compounds for binding activity to the proteins. Several methods of automating assays have been developed in recent years so as to permit screening of tens of thousands of compounds in a short period. See, e.g., Fodor, *et al.* (1991) *Science 251*:767-773 which describes means for testing of binding affinity by a plurality of defined polymers synthesized on a solid substrate. The development of suitable assays can be greatly facilitated by the availability of large amounts of purified, soluble Flt3 ligand as provided by this invention.

For example, antagonists can normally be found once the ligand has been structurally defined. Testing of potential ligand analogs is now possible upon the development of highly automated assay methods using a purified Flt3 receptor. In particular, new agonists and antagonists will be discovered by using screening techniques described herein. Of particular importance are compounds found to have a combined binding

affinity for multiple Flt3 receptors, e.g., compounds which can serve as antagonists for species variants of Flt3 ligand.

This invention is particularly useful for screening compounds by using recombinant receptor in any of a variety of drug screening techniques. The advantages of using a recombinant protein in screening for specific ligands include: (a) improved renewable source of the Flt3 receptor from a specific source; (b) potentially greater number of ligands per cell giving better signal to noise ratio in assays; and (c) species variant specificity (theoretically giving greater biological and disease specificity).

One method of drug screening utilizes eukaryotic or prokaryotic host cells which are stably transformed with recombinant DNA molecules expressing the Flt3 receptor. Cells may be isolated which express a receptor in isolation from any others. Such cells, either in viable or fixed form, can be used for standard ligand/receptor binding assays. See also, Parce, *et al.* (1989) *Science 246*:243-247; and Owicki, *et al.* (1990) *Proc. Nat'l Acad. Sci.* USA *87*:4007-4011 describe sensitive methods to detect cellular responses.

Competitive assays are particularly useful, where the cells (source of Flt3 ligand) are contacted and incubated with a labeled receptor or antibody having known binding affinity to the ligand, such as ^{125}I-antibody, and a test sample whose binding affinity to the binding composition is being measured. The bound and free labeled binding compositions are then separated to assess the degree of ligand binding.

The amount of test compound bound is inversely proportional to the amount of labeled receptor binding to the known source. Any one of numerous techniques can be used to separate bound from free ligand to assess the degree of ligand binding. This separation step could typically involve a procedure such as adhesion to filters followed by washing, adhesion to plastic followed by washing, or centrifugation of the cell membranes. Viable cells could also be used to screen

for the effects of drugs on Flt3 ligand mediated functions, e.g., second messenger levels, i.e., Ca^{++}; cell proliferation; inositol phosphate pool changes; and others. Some detection methods allow for elimination of a separation step, e.g., a proximity sensitive detection system. Calcium sensitive dyes will be useful for detecting Ca^{++} levels, with a fluorimeter or a fluorescence cell sorting apparatus.

Another method utilizes membranes from transformed eukaryotic or prokaryotic host cells as the source of the Flt3 ligand. These cells are stably transformed with DNA vectors directing the expression of a Flt3 ligand, e.g., an engineered membrane bound form. Essentially, the membranes would be prepared from the cells and used in any receptor/ligand binding assay such as the competitive assay set forth above.

Still another approach is to use solubilized, unpurified or solubilized, purified Flt3 ligand from transformed eukaryotic or prokaryotic host cells. This allows for a "molecular" binding assay with the advantages of increased specificity, the ability to automate, and high drug test throughput.

Another technique for drug screening involves an approach which provides high throughput screening for compounds having suitable binding affinity to Flt3 receptor and is described in detail in Geysen, European Patent Application 84/03564, published on September 13, 1984. First, large numbers of different small peptide test compounds are synthesized on a solid substrate, e.g., plastic pins or some other appropriate surface. Then all the pins are reacted with solubilized, unpurified or solubilized, purified Flt3 receptor, and washed. The next step involves detecting bound Flt3 receptor.

Rational drug design may also be based upon structural studies of the molecular shapes of the Flt3 ligand and other effectors or analogs. Effectors may be other proteins which mediate other functions in response to ligand binding, or other proteins which normally interact with the receptor. One

means for determining which sites interact with specific other proteins is a physical structure determination, e.g., x-ray crystallography or 2 dimensional NMR techniques. These will provide guidance as to which amino acid residues form molecular contact regions. For a detailed description of protein structural determination, see, e.g., Blundell and Johnson (1976) *Protein Crystallography*, Academic Press, New York.

Purified Flt3 ligand can be coated directly onto plates for use in the aforementioned drug screening techniques. However, non-neutralizing antibodies to these ligands can be used as capture antibodies to immobilize the respective ligand on the solid phase.

Kits

This invention also contemplates use of Flt3 ligand proteins, fragments thereof, peptides, and their fusion products in a variety of diagnostic kits and methods for detecting the presence of ligand or a Flt3 receptor. Typically the kit will have a compartment containing either a defined Flt3 ligand peptide or gene segment or a reagent which recognizes one or the other, e.g., receptor fragments or antibodies.

A kit for determining the binding affinity of a test compound to a Flt3 ligand would typically comprise a test compound; a labeled compound, for example a receptor or antibody having known binding affinity for the ligand; a source of Flt3 ligand (naturally occurring or recombinant); and a means for separating bound from free labeled compound, such as a solid phase for immobilizing the ligand. Once compounds are screened, those having suitable binding affinity to the ligand can be evaluated in suitable biological assays, as are well known in the art, to determine whether they act as agonists or antagonists to the receptor. The

availability of recombinant Flt3 ligand polypeptides also provide well defined standards for calibrating such assays.

A preferred kit for determining the concentration of, for example, a Flt3 ligand in a sample would typically comprise a labeled compound, e.g., receptor or antibody, having known binding affinity for the ligand, a source of ligand (naturally occurring or recombinant) and a means for separating the bound from free labeled compound, for example, a solid phase for immobilizing the Flt3 ligand. Compartments containing reagents, and instructions, will normally be provided.

Antibodies, including antigen binding fragments, specific for the Flt3 ligand or ligand fragments are useful in diagnostic applications to detect the presence of elevated levels of Flt3 ligand and/or its fragments. Such diagnostic assays can employ lysates, live cells, fixed cells, immunofluorescence, cell cultures, body fluids, and further can involve the detection of antigens related to the ligand in serum, or the like. Diagnostic assays may be homogeneous (without a separation step between free reagent and antigen-ligand complex) or heterogeneous (with a separation step). Various commercial assays exist, such as radioimmunoassay (RIA), enzyme-linked immunosorbent assay (ELISA), enzyme immunoassay (EIA), enzyme-multiplied immunoassay technique (EMIT), substrate-labeled fluorescent immunoassay (SLFIA), and the like. For example, unlabeled antibodies can be employed by using a second antibody which is labeled and which recognizes the antibody to a Flt3 ligand or to a particular fragment thereof. Similar assays have also been extensively discussed in the literature. See, e.g., Harlow and Lane (1988) *Antibodies: A Laboratory Manual*, CSH.

Anti-idiotypic antibodies may have similar use to diagnose presence of antibodies against a Flt3 ligand, as such may be diagnostic of various abnormal states. For example, overproduction of Flt3 ligand may result in production of various immunological reactions which may be diagnostic of

abnormal physiological states, particularly in proliferative cell conditions such as cancer or abnormal differentiation.

Frequently, the reagents for diagnostic assays are supplied in kits, so as to optimize the sensitivity of the assay. For the subject invention, depending upon the nature of the assay, the protocol, and the label, either labeled or unlabeled antibody or receptor, or labeled Flt3 ligand is provided. This is usually in conjunction with other additives, such as buffers, stabilizers, materials necessary for signal production such as substrates for enzymes, and the like. Preferably, the kit will also contain instructions for proper use and disposal of the contents after use. Typically the kit has compartments for each useful reagent. Desirably, the reagents are provided as a dry lyophilized powder, where the reagents may be reconstituted in an aqueous medium providing appropriate concentrations of reagents for performing the assay.

Any of the aforementioned constituents of the drug screening and the diagnostic assays may be used without modification or may be modified in a variety of ways. For example, labeling may be achieved by covalently or non-covalently joining a moiety which directly or indirectly provides a detectable signal. In any of these assays, the ligand, test compound, Flt3 ligand, or antibodies thereto can be labeled either directly or indirectly. Possibilities for direct labeling include label groups: radiolabels such as ^{125}I, enzymes (U.S. Pat. No. 3,645,090) such as peroxidase and alkaline phosphatase, and fluorescent labels (U.S. Pat. No. 3,940,475) capable of monitoring the change in fluorescence intensity, wavelength shift, or fluorescence polarization. Possibilities for indirect labeling include biotinylation of one constituent followed by binding to avidin coupled to one of the above label groups.

There are also numerous methods of separating the bound from the free ligand, or alternatively the bound from the free test compound. The Flt3 ligand can be immobilized on

various matrixes followed by washing. Suitable matrixes include plastic such as an ELISA plate, filters, and beads. Methods of immobilizing the Flt3 ligand to a matrix include, without limitation, direct adhesion to plastic, use of a capture antibody, chemical coupling, and biotin-avidin. The last step in this approach involves the precipitation of ligand/receptor or ligand/antibody complex by any of several methods including those utilizing, e.g., an organic solvent such as polyethylene glycol or a salt such as ammonium sulfate. Other suitable separation techniques include, without limitation, the fluorescein antibody magnetizable particle method described in Rattle, *et al.* (1984) *Clin. Chem. 30*:1457-1461, and the double antibody magnetic particle separation as described in U.S. Pat. No. 4,659,678.

Methods for linking proteins or their fragments to the various labels have been extensively reported in the literature and do not require detailed discussion here. Many of the techniques involve the use of activated carboxyl groups either through the use of carbodiimide or active esters to form peptide bonds, the formation of thioethers by reaction of a mercapto group with an activated halogen such as chloroacetyl, or an activated olefin such as maleimide, for linkage, or the like. Fusion proteins will also find use in these applications.

Another diagnostic aspect of this invention involves use of oligonucleotide or polynucleotide sequences taken from the sequence of a Flt3 ligand. These sequences can be used as probes for detecting levels of the ligand message in samples from patients suspected of having an abnormal condition, e.g., cancer or developmental problem. The preparation of both RNA and DNA nucleotide sequences, the labeling of the sequences, and the preferred size of the sequences has received ample description and discussion in the literature. Normally an oligonucleotide probe should have at least about 14 nucleotides, usually at least about 18 nucleotides, and the

polynucleotide probes may be up to several kilobases. Various labels may be employed, most commonly radionuclides, particularly ^{32}P.

However, other techniques may also be employed, such as using biotin modified nucleotides for introduction into a polynucleotide. The biotin then serves as the site for binding to avidin or antibodies, which may be labeled with a wide variety of labels, such as radionuclides, fluorescers, enzymes, or the like. Alternatively, antibodies may be employed which can recognize specific duplexes, including DNA duplexes, RNA duplexes, DNA-RNA hybrid duplexes, or DNA-protein duplexes. The antibodies in turn may be labeled and the assay carried out where the duplex is bound to a surface, so that upon the formation of duplex on the surface, the presence of antibody bound to the duplex can be detected. The use of probes to the novel anti-sense RNA may be carried out in any conventional techniques such as nucleic acid hybridization, plus and minus screening, recombinational probing, hybrid released translation (HRT), and hybrid arrested translation (HART). This also includes amplification techniques such as polymerase chain reaction (PCR).

Diagnostic kits which also test for the qualitative or quantitative presence of other markers are also contemplated. Diagnosis or prognosis may depend on the combination of multiple indications used as markers. Thus, kits may test for combinations of markers. See, e.g., Viallet, *et al.* (1989) *Progress in Growth Factor Res.* 1:89-97.

EXAMPLES

The broad scope of this invention is best understood with reference to the following examples, which are not intended to illustrate but not to limit the invention to specific embodiments.

General Methods

Some of the standard methods are described or referenced, e.g., in Maniatis, *et al.* (1982) *Molecular Cloning, A Laboratory Manual*, Cold Spring Harbor Laboratory, Cold Spring Harbor Press; Sambrook, *et al.* (1989) *Molecular Cloning: A Laboratory Manual*, (2d ed.), vols 1-3, CSH Press, NY; Ausubel, *et al.*, *Biology*, Greene Publishing Associates, Brooklyn, NY; or Ausubel, *et al.* (1987 and Supplements) *Current Protocols in Molecular Biology*, Greene/Wiley, New York; Innis, *et al.* (eds.)(1990) *PCR Protocols: A Guide to Methods and Applications* Academic Press, N.Y.

Methods for protein purification include such methods as ammonium sulfate precipitation, column chromatography, electrophoresis, centrifugation, crystallization, and others. See, e.g., Ausubel, *et al.* (1987 and periodic supplements); Deutscher (1990) "Guide to Protein Purification" in *Methods in Enzymology*, vol. 182, and other volumes in this series; and manufacturer's literature on use of protein purification products, e.g., Pharmacia, Piscataway, N.J., or Bio-Rad, Richmond, CA. Combination with recombinant techniques allow fusion to appropriate segments, e.g., to a FLAG sequence or an equivalent which can be fused via a protease-removable sequence. See, e.g., Hochuli (1989) *Chemische Industrie 12*:69-70; Hochuli (1990) "Purification of Recombinant Proteins with Metal Chelate Absorbent" in Setlow (ed.) *Genetic Engineering, Principle and Methods* 12:87-98, Plenum Press, N.Y.; and Crowe, *et al.* (1992) *QIAexpress: The High Level Expression & Protein Purification System* QUIAGEN, Inc., Chatsworth, CA.

FACS analyses are described in Melamed, *et al.* (1990) *Flow Cytometry and Sorting* Wiley-Liss, Inc., New York, NY; Shapiro (1988) *Practical Flow Cytometry* Liss, New York, NY; and Robinson, *et al.* (1993) *Handbook of Flow Cytometry Methods* Wiley-Liss, New York, NY.

Production and Sequencing of the Flt3 Ligand

Assays for the Flt3/Flk2 ligand

Baf3 cells (mouse pre-B cell line; see Palacios, *et al.* (1985) *Cell 41*:727-734; and Palacios, *et al.* (1984) *Nature 309*:126-131) were stably transfected with the cDNA for the Flt3 receptor, Rosnet, *et al.* (1991) *Oncogene 6*:1641-1650. Two methods were used to observe the response of these cells to samples containing the ligand. The main assay is an MTT assay in which the Flt3-transfected cells (Baflts) survive a 24-hour incubation in the presence of the ligand (but not if the ligand is absent) and can then take up and cleave the MTT dye. See Mosmann (1983) *J. Immunol. Methods 65*:55-63. Untransfected Baf cells die both in the presence and absence of the ligand, and samples are therefore routinely assayed in parallel on both cell types. The difference in signal obtained from these two cell types (Baflt-Baf) is a measure of the amount of ligand present in a sample.

In some cases where active samples also contain substances that are toxic to the cells, the MTT assay with Baflts can fail to detect the presence of the ligand. A second method for detecting the ligand in these cases is to take advantage of the fact that receptors such as c-fms, c-kit, and flt-3 rapidly (within 5 minutes) auto-phosphorylate themselves while binding their appropriate ligands. This response is much less sensitive to toxic substances than is the 24-hour MTT assay.

Auto-phosphorylated receptor is observed by lysing the responder cells with detergent, e.g., NP-40, immunoprecipitating Flt3 using antibodies directed against the intracellular portion of the molecule, and separating the immunoprecipitate on SDS-PAGE, blotting to nitrocellulose, probing with an anti-phosphotyrosine monoclonal antibody, and detecting phosphotyrosine, e.g., with a horseradish

peroxidase-linked anti-immunoglobulin and developing with a chemiluminescent substrate.

Source of the Flt3 Ligand

Multiple cell lines were screened for one which expresses a Flt3 ligand. A mouse thymic stromal cell line TA4 (provided by Donna Rennick, DNAX Research Institute) was selected for its ease in handling for large scale production. Cells were grown on roller bottles and 7 day-conditioned media supernatants (serum-free) were harvested, passed through 0.22 μm filters, concentrated 100-fold, and stored frozen at -80° C.

Biochemical Characterization of the Flt3 Ligand.

The Flt3 ligand activity has been defined by its separation parameters using different protein separation techniques. However, because of low expression levels, the detection of the activity was greatly facilitated by the concentration of the media. Aliquots of 100x concentrated TA4 cell supernatant, typically representing 10 L of crude supernatant, were subjected to various biochemical purification techniques including ammonium sulfate precipitation, hydrophobic interaction chromatography, anion and cation exchange chromatography, gel filtration chromatography, and reversed phase chromatography. See Table 2. The behavior of the biological activity representing the Flt3 ligand in each of these techniques is summarized below:

Ammonium Sulfate precipitation (at 4° C): activity found in 60-85% saturated $(NH_4)_2SO_4$ pellet;

Hydrophobic Interaction Chromatography [$(NH_4)_2SO_4$ gradient in 20 mM Tris, pH 7.5 on a Phenyl-5PW column]: activity eluted between 900-750 mM $(NH_4)_2SO_4$;

Anion Exchange Chromatography (NaCl gradient in 20 mM Tris, pH 7.5 on Mono Q column): activity eluted between 130-250 mM NaCl;

Cation Exchange Chromatography (NaCl gradient in 10 mM citrate, pH 3.0 on Mono S column): the bulk of the activity eluted between 440-540 mM NaCl;

Gel Filtration (SEPHACRYL® S200 column): the activity ran with an apparent molecular weight of 70 kD;

Reversed Phase HPLC (water to acetonitrile gradient in 0.1% TFA on a Poros R/H column): the activity eluted between 32-35% acetonitrile.

Engineering, Production and Purification of Soluble Flt3

A soluble fragment of the Flt3 receptor was constructed by removing the membrane spanning and cytoplasmic domains of the Flt3 receptor, fused to a sequence, e.g., FLAG, useful for purifying the expression product of the construct. See, e.g., Crowe, *et al.* (1992) *QIAexpress: The High Level Expression & Protein Purification System* QUIAGEN, Inc. Chatsworth, CA; and Hopp, *et al.* (1988) *Bio/Technology* *6*:1204-1210. The sequence allows for efficient affinity purification of the soluble product. Appropriate secretion or processing sites may also be engineered into the construct by standard methods. Purification may be achieved by use of affinity purification, e.g., antibodies against the receptor, or by standard protein purification methods. Typically, the affinity reagents or purification procedures can be performed using recombinant receptor.

More specifically, two tags were engineered to the carboxy terminus of the extracellular domain of the Flt3 receptor. pMEXneo-Flt3 was used as a source to modify the

Flt3 cDNA to introduce a BglII site at nucleotide 1662, directly following Ser544, the last amino acid of the extracellular domain. An XmaI/BglII fragment containing the entire extracellular Flt3 domain was cloned into pHFBgl, a derivative of pVL1393 from Invitrogen Corp. The pHFBgl contains a His_6-FLAG-stop codon sequence fused in frame with the BglII site of the polylinker. See Table 4. The resulting plasmid was named pHF/Flt3.

Table 4. Relevant Portion of Soluble Flt3 Receptor Construct

```
        Xma I
        Sma I                          Not I
   BamHI            XbaI EcoRI      XmaIIIPstIBglII
5' GGATCCCGGGTACCTTCTAGAATTCCGGAGCGGCCGCTGCAGATCTCAT-
3' CCTAGGGCCCATGGAAGATCTTAAGGCCTCGCCGGCGACGTCTAGAGTA-
    AspProGlyTyrLeuLeuGluPheArgSerGlyArgCysArgSerHis

  CACCATCACCATCACGATTACAAGGACGATGACGATAAGTAATGA 3'
   GTGGTAGTGGTAGTGCTAATGTTCCTGCTACTGCTATTCATTACT 5'
   HisHisHisHisHisAspTyrLysAspAspAspAspLysStpStp
```

The nucleotide and amino acid sequences shown in Table 4 are also defined in the Sequence Listing by SEQ ID NOs: 36 and 37, respectively.

Sf9 insect cells were transfected with pHF/Flt and a virus stock was prepared. After infection of Sf9 cells with recombinant virus, medium was collected. The soluble secreted Flt3-His_6-FLAG expression product was purified from the medium using a nickel-NTA resin from Qiagen. The purified Flt3 receptor was coupled to an M2 anti-FLAG antibody column. See below.

Purification of the Flt3 Ligand

The Flt3 ligand was isolated by a combination of affinity chromatography using the Flt3 receptor as a specific binding

reagent in combination with the earlier defined physical properties allowing separation from other proteins and contaminants. Similar techniques using human cell assays and human cell sources could be applied to isolate a human ligand.

100L of crude TA4 supernatant was buffer exchanged into PBS and concentrated to 1000x. This was then tumbled overnight with 2 ml M2 (anti-FLAG) beads that had been pre-loaded with soluble Flt3. The beads were then washed with several column volumes of PBS, and the bound material was eluted with 3 ml 100 mM glycine, pH 2.5. The eluate was collected into a tube containing sufficient 2 M Tris, pH 7.5 to neutralize the glycine. The neutralized eluate was then loaded onto a 4.6 x 100 mm Poros R/H column and the proteins chromatographed with a linear water/acetonitrile gradient in 0.1% TFA.

Samples of each fraction were dried down for bioassay and SDS-PAGE. The molecule representing the Flt3 ligand activity ran on reduced SDS-PAGE as an apparently glycosylated protein at approximately 30 kilodaltons. Fractions containing the bulk of the biological activity were dried down completely and combined into sufficient Laemmli gel sample buffer (containing DTT) to be run in a single lane on a 12% mini-gel (SDS-PAGE). The gel was stained with Coomassie blue, destained, and the band representing the Flt3 ligand was carefully excised. This slice contained biochemically pure Flt3 ligand. 100 L of TA4 cell supernatant contained less than 5 micrograms of this protein. The purified protein has a specific activity of 1×10^7 Units per milligram on the Baf assay. One unit is that which provides half maximum stimulation in a 100 µl assay.

Generation and Purification of Flt3 Ligand Tryptic Peptides

The prep gel slice containing the ligand was briefly rinsed with water and acetonitrile to remove excess SDS, smashed into tiny fragments, taken to dryness under vacuum

on a SPEEDVAC® (Savant), and then solubilized in 0.2 ml Tris buffer (pH 7.5) containing 0.5 µg modified trypsin (a chemically modified form that will not digest itself) plus 2 mM DTT, and 0.01% Tween 20. The cleavage reaction was carried out at 37° C for 6 hr, at which time a second 0.5 µg aliquot of trypsin was added, and the digestion continued overnight. The reaction mix was spun at 13 K and loaded onto a 2.1 x 100 mm AQUAPORE® RP-300 reversed phase column, and peptides eluted with a linear 4-44% acetonitrile gradient (with constant 0.1% TFA). In some cases peptides were rechromatographed on the same column with a 16 - 44% acetonitrile gradient (with constant 0.1 mM heptafluorobutyric acid (HFBA)). Eluting peptides were monitored at both 215 nm and 280 nm and were collected by hand.

Determination of the Amino Acid Sequence of Peptides of the Flt3 Ligand

Peptide sequences were determined using an Applied Biosystems 477A Sequencer. Fragments provided peptide sequences of Table 1 reconstructed into consensus sequences; peptide 17 is the amino terminus:

12. FVQTXISHLLK

13. DYPVTVAVNLQ

14. TPDAYFSHSPISSNFKVKFRELTVHLLK

15. WIEQLK

16. ILFXLFAQYR

17. TPDCY FSHSP ISSNF KVKFR ELTVH LLKDY PVTVA VNLQD EK

Isolation of a DNA Clone Encoding Flt3 Ligand

The purified protein or defined peptides are useful for generating antibodies by standard methods, as described above. Synthetic peptides or purified protein are presented to an immune system to generate monoclonal or polyclonal antibodies. See, e.g., Coligan (1991) *Current Protocols in Immunology* Wiley/Greene; and Harlow and Lane (1989)

Antibodies: A Laboratory Manual Cold Spring Harbor Press. Alternatively, the Flt3 receptor is used as a specific binding reagent, and advantage can be taken of its specificity of binding, much like an antibody would be used. In either case, the binding reagent is either labeled as described above, e.g., fluorescence or otherwise, or immobilized to a substrate for panning methods.

The binding composition is used for screening of an expression library made from a cell line which expresses a Flt3 ligand. Standard staining techniques are used to detect or sort intracellular or surface expressed ligand, or surface expressing transformed cells are screened by panning. Screening of intracellular expression is performed by various staining or immunofluorescence procedures. See also McMahan, *et al.* (1991) *EMBO J.* 10:2821-2832.

For example, on day 0, precoat 2-chamber PERMANOX® slides with 1 ml per chamber of fibronectin, 10 ng/ml in PBS, for 30 min at room temperature. Rinse once with PBS. Then plate COS cells at 2-3 x 10^5 cells per chamber in 1.5 ml of growth media. Incubate overnight at 37° C.

On day 1 for each sample, prepare 0.5 ml of a solution of 66 µg/ml DEAE-dextran, 66 µM chloroquine, and 4 µg DNA in serum free DME. For each set, a positive control is prepared, e.g., of huIL-10-FLAG cDNA at 1 and 1/200 dilution, and a negative mock. Rinse cells with serum free DME. Add the DNA solution and incubate 5 hr at 37° C. Remove the medium and add 0.5 ml 10% DMSO in DME for 2.5 min. Remove and wash once with DME. Add 1.5 ml growth medium and incubate overnight.

On day 2, change the medium. On days 3 or 4, the cells are fixed and stained. Rinse the cells twice with Hank's Buffered Saline Solution (HBSS) and fix in 4% paraformaldehyde (PFA)/glucose for 5 min. Wash 3X with HBSS. The slides may be stored at -80° C after all liquid is removed. For each chamber, 0.5 ml incubations are performed

as follows. Add HBSS/saponin(0.1%) with 32 µl/ml of 1M NaN_3 for 20 min. Cells are then washed with HBSS/saponin 1X. Soluble Flt3 receptor/antibody complex to cells and incubate for 30 min. Wash cells twice with HBSS/saponin. Add second antibody, e.g., Vector anti-mouse antibody, at 1/200 dilution, and incubate for 30 min. Prepare ELISA solution, e.g., Vector Elite ABC horseradish peroxidase solution, and preincubate for 30 min. Use, e.g., 1 drop of solution A (avidin) and 1 drop solution B (biotin) per 2.5 ml HBSS/saponin. Wash cells twice with HBSS/saponin. Add ABC HRP solution and incubate for 30 min. Wash cells twice with HBSS, second wash for 2 min, which closes cells. Then add Vector diaminobenzoic acid (DAB) for 5 to 10 min. Use 2 drops of buffer plus 4 drops DAB plus 2 drops of H_2O_2 per 5 ml of glass distilled water. Carefully remove chamber and rinse slide in water. Air dry for a few minutes, then add 1 drop of Crystal Mount and a cover slip. Bake for 5 min at 85-90° C.

Alternatively, the binding compositions are used to affinity purify or sort out cells expressing the ligand. See, e.g., Sambrook, *et al.* or Ausubel *et al.*

In another method, the peptide segments are used to predict appropriate oligonucleotides to screen a library. The genetic code is used to select appropriate oligonucleotides useful as probes for screening a library. See, e.g., Table 3. In combination with polymerase chain reaction (PCR) techniques, synthetic oligonucleotides in appropriate orientations are used as primers to select correct clones from a library. Various combinations of upstream/downstream sense/antisense combinations are tested until an appropriate clone is amplified and detected.

For example, a functional screening approach is applied based upon a biological assay for the ligand. The Baf pro-B cell line is transfected with the receptor cDNA to generate a stable transfectant. The transfectant exhibits a mitogenic

response to the TA4 stromal cell supernatants, especially detectable when the supernatant is concentrated 100 X. The parental transfectant cells are non-responsive to the supernatants. An expression cDNA library made from the producing TA4 cells is made. Pools of random cDNA clones are transfected into COS monkey cells and supernatants are screened for ligand biological activity. Positive pools are subdivided to isolate single clones.

In another approach, expression screening is based upon a biochemical assay for a soluble ligand. The assay is based upon the autophosphorylation activity rather than the mitogenic activity. Total cell lysates of the exposed test cells are prepared and immunoprecipitated with anti-phosphotyrosine antiserum. The immunoprecipitate is run on a polyacrylamide gel, and is, e.g., blotted to a filter and developed with antibody staining of phosphotyrosine.

In yet another approach, functional screening of a membrane bound ligand is by either mitogenic bioassay or biochemical autophosphorylation assay. Appropriate fusion vectors can produce an appropriate expression construct.

Another strategy is to screen for a membrane bound ligand by panning. The receptor cDNA is constructed as described above. The soluble receptor or antibodies raised against the defined peptide fragments can be immobilized and used to immobilize expressing cells. Immobilization may be achieved by use of appropriate antibodies which recognize, e.g., the FLAG sequence of the soluble receptor construct, or by use of antibodies raised against the first antibodies. Recursive cycles of selection and amplification lead to enrichment of appropriate clones and eventual isolation of ligand expressing clones.

Phage expression libraries can be screened by soluble receptor or anti-fragment antibodies. Appropriate label techniques, e.g., anti-FLAG antibodies, will allow specific labeling of appropriate clones.

Screening by hybridization using degenerate probes based upon the peptide sequences will also allow isolation of appropriate clones. Alternatively, use of appropriate primers for PCR screening will yield enrichment of appropriate nucleic acid clones.

Similar methods are applicable to isolate either species or allelic variants. Species variants are isolated using cross-species hybridization techniques based upon isolation of a full length isolate or fragment from one species as a probe.

Alternatively, similar assays can be developed, e.g., in human cells for isolation of the Flt3 ligand activity. The mouse cell assays detect a human Flt3 ligand activity, e.g., in human thymic epithelial SV48 cells.

PCR amplification using degenerate primers based upon vector and downstream sequences generated products in a first round of PCR amplification. Primers based upon a vector sequence and degenerate primers from the NLQDEK peptide sequence were used in the first round of amplification. The product of the first amplification was subjected to a second round of PCR amplification using degenerate primers based upon TPDCYF and YPVTVAV peptide sequences. The product of this second round of PCR amplification resulted in a 108 bp product probed using probes based upon NFKVKF sequence. The length is consistent with that predicted by the provided sequence. Sequencing of this 108 bp product provided the nucleotide sequence:

ACT CCT GAC TGT TAC TTC AGC CAC AGT CCC ATC TCC TCC AAC TTC AAA GTG AAG TTT AGA GAG TTG ACT GAC CAC CTG CTT AAA GAT,

which encodes the expected peptide sequence. Further sequencing of a clone has provided the sequence of Table 5.

Table 5: Nucleotide and Amino Acid Sequence from a Flt3 Ligand Fragment Clone

```
ACTCCTGACTGTTACTTCAGCCACAGTCCCATCTCCTCCAACTTCAAAGTGAAGTTTAGAGAGTTGACT
THRPROASPCYSTYRPHESERHISSERPROILESERSERASNPHELYSVALLYSPHEARGGLULEUTHR

GACCACCTGCTTAAAGATTACCCAGTCACTGTGGCCGTCAATCTTCAGGACGAGAAGCACTGCAAGGCC
ASPHISLEULEULYSASPTYRPROVALTHRVALALAVALASNLEUGLNASPGLULYSHISCYSLYSALA

TTGTGGAGCCTCTTCCTAGCCCAGCGCTGGATAGAGCAACTGAAGACTGTGGCAGGGTCTAAGATGCAA
LEUTRPSERLEUPHELEUALAGLNARGTRPILEGLUGLNLEULYSTHRVALALAGLYSERLYSMETGLN

ACGCTTCTGGAGGACGTCAACACCGAGATACATTTTGTCACCTCATGTACCTTCCAGCCCCTACCAGAA
THRLEULEUGLUASPVALASNTHRGLUILEHISPHEVALTHRSERCYSTHRPHEGLNPROLEUPROGLU

TGTCTGCGATTCGTACAGACCAATATA
CYSLEUARGPHEVALGLNTHRASNILE
```

The sequences shown in Table 5, which are also defined in the Sequence Listing by SEQ ID NO: 18, eliminate degeneracy of the relevant encoding nucleic acid sequence. This probe is used to screen longer or full length libraries, e.g., from mouse or other mammalian species. Allelic variants and other related genes will also be isolatable using, e.g., hybridization techniques. See Sambrook, *et al.* and Ausubel, *et al.*

Isolation of a DNA Clone Encoding the Human Flt3 Ligand.

A human 29SV48 stromal cell cDNA library in pME18S, constructed from poly A+ mRNA, was screened with an 800 bp DNA fragment derived from the mouse T118 clone. This fragment encompasses the coding region conserved between the two mouse clones, T118 and T110 (see Table 3). Hybridization of filters with ^{32}P labeled probe was carried out in a solution of 20% formamide, 6 X SSPE, 5 X Denhardt's, 100 µg/ml tRNA, and 0.1% SDS at 42° C overnight. Filters were washed two times at room temperature with 2 X SSC and 0.1% SDS for 10-15 minutes. Filters were then washed in 0.1 X SSPE, 0.1% SDS under one of the following conditions: three

times at 50° C for 30 min each; three times at 55° C for 30 min each; once at 55° C for 30 min and once at 60° C for 30 min; or once at 60° C for 30 min.

Approximately 20 positive colonies were selected using these conditions. These were partially sequenced and two clones, S86 and S109 were found to be approximately 75% homologous to the mouse clones over the first 163 amino acids. Clone S86 continued to show homology to clone T110 until the stop codon, although to a lesser degree, for an overall homology of 66%. Clones T118 and S109 do not show homology to each other or the other clones after mouse residue 163 (human residue 160). An additional mouse clone designated MB8 has a 29 amino acid insert at the junction between the common and divergent portions of the mouse ligand.

Many modifications and variations of this invention can be made without departing from its spirit and scope, as will be apparent to those skilled in the art. The specific embodiments described herein are offered by way of example only, and the invention is to be limited only by the terms of the appended claims, along with the full scope of equivalents to which such claims are entitled.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT:
(A) NAME: Schering Corporation
(B) STREET: One Giralda Farms
(C) CITY: Madison
(D) STATE: New Jersey
(E) COUNTRY: U.S.A.
(F) POSTAL CODE (ZIP): 07940-1000
(G) TELEPHONE: 201-822-7375
(H) TELEFAX: 201-822-7039
(I) TELEX: 219165

(i) APPLICANT:
(A) NAME: Institut National De La Sante Et De La Recherche Medicale
(B) STREET: 101 rue de Tolbiac
(C) CITY: 75643 Paris
(D) STATE: Cedex 13
(E) COUNTRY: France
(F) POSTAL CODE (ZIP):
(G) TELEPHONE: 33 1 44 23 60 41
(H) TELEFAX: 33 1 45 85 07 66
(I) TELEX:

(ii) TITLE OF INVENTION: Purified Mammalian Flt3 Ligands and Agonists and Antagonists Thereof

(iii) NUMBER OF SEQUENCES: 37

(iv) COMPUTER READABLE FORM:

(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: Apple Macintosh
(C) OPERATING SYSTEM: Macintosh 6.0.5
(D) SOFTWARE: Microsoft Word 5.1a

(v) CURRENT APPLICATION DATA:
(A) APPLICATION NUMBER:
(B) FILING DATE:

(vi) PRIOR APPLICATION DATA:
(A) APPLICATION NUMBER: US 08/162,413
(B) FILING DATE: 03-DEC-1993

(vi) PRIOR APPLICATION DATA:
(A) APPLICATION NUMBER: US 08/155,111
(B) FILING DATE: 19-NOV-1993

(vi) PRIOR APPLICATION DATA:
(A) APPLICATION NUMBER: US 08/112,391
(B) FILING DATE: 24-AUG-1993

(vi) PRIOR APPLICATION DATA:
(A) APPLICATION NUMBER: US 08/106,340
(B) FILING DATE: 13-AUG-1993

(vi) PRIOR APPLICATION DATA:
(A) APPLICATION NUMBER: US 08/092,549
(B) FILING DATE: 16-JUL-1993

(vi) PRIOR APPLICATION DATA:
(A) APPLICATION NUMBER: US 08/089,263
(B) FILING DATE: 07-JUL-1993

(vi) PRIOR APPLICATION DATA:
(A) APPLICATION NUMBER: US 08/065,231
(B) FILING DATE: 19-MAY-1993

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 11 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

```
Phe Val Gln Thr Xaa Ile Ser His Leu Leu Lys
1               5                  10
```

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 14 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

```
Asp Tyr Pro Val Thr Val Ala Val Asn Leu Gln Asp Glu Lys
1               5                  10
```

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 16 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

```
Thr Pro Asp Xaa Tyr Phe Ser His Ser Pro Ile Ser Ser Asn Phe Lys
1               5                  10                  15
```

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 10 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

```
Trp Ile Glu Gln Leu Lys Gln Pro Gly Ser
1               5                  10
```

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 8 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

```
Glu Leu Thr Val His Leu Leu Lys
1               5
```

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 10 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

```
Ile Leu Phe Xaa Leu Phe Leu Gln Tyr Arg
1               5                  10
```

(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 9 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

```
Ser His Ser Pro Ile Ser Ser Asn Phe
1               5
```

(2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 6 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

```
Trp Ile Glu Gln Leu Lys
1               5
```

(2) INFORMATION FOR SEQ ID NO:9:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 11 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

```
Asp Tyr Pro Val Thr Val Ala Val Asn Leu Gln
1               5                   10
```

(2) INFORMATION FOR SEQ ID NO:10:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 22 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

```
Asp Ala Tyr Phe Ser His Ser Pro Ile Ser Ser Asn Phe Lys Val Lys
1               5                   10                  15

Phe Arg Glu Leu Thr Val
            20
```

(2) INFORMATION FOR SEQ ID NO:11:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 8 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

```
Asp Tyr Pro Val Thr Val Ala Ala
1               5
```

(2) INFORMATION FOR SEQ ID NO:12:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 28 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

```
Thr Pro Asp Ala Tyr Phe Ser His Ser Pro Ile Ser Ser Asn Phe Lys
1               5                   10                  15

Val Lys Phe Arg Glu Leu Thr Val His Leu Leu Lys
            20                  25
```

(2) INFORMATION FOR SEQ ID NO:13:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 6 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

```
Trp Ile Glu Gln Leu Lys
1               5
```

(2) INFORMATION FOR SEQ ID NO:14:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 11 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:

```
Phe Val Gln Thr Xaa Ile Ser His Leu Leu Lys
1               5                   10
```

(2) INFORMATION FOR SEQ ID NO:15:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 10 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:15:

```
Ile Leu Phe Xaa Leu Phe Ala Gln Tyr Arg
1               5                   10
```

(2) INFORMATION FOR SEQ ID NO:16:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 11 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:16:

```
Asp Tyr Pro Val Thr Val Ala Val Asn Leu Gln
1               5                   10
```

(2) INFORMATION FOR SEQ ID NO:17:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 42 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:17:

```
Thr Pro Asp Cys Tyr Phe Ser His Ser Pro Ile Ser Ser Asn Phe Lys
1               5                   10                  15

Val Lys Phe Arg Glu Leu Thr Val His Leu Leu Lys Asp Tyr Pro Val
            20                  25                  30

Thr Val Ala Val Asn Leu Gln Asp Glu Lys
        35                  40
```

(2) INFORMATION FOR SEQ ID NO:18:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 303 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:18:

```
ACT CCT GAC TGT TAC TTC AGC CAC AGT CCC ATC TCC TCC AAC TTC AAA     48
Thr Pro Asp Cys Tyr Phe Ser His Ser Pro Ile Ser Ser Asn Phe Lys
  1               5                  10                  15

GTG AAG TTT AGA GAG TTG ACT GAC CAC CTG CTT AAA GAT TAC CCA GTC     96
Val Lys Phe Arg Glu Leu Thr Asp His Leu Leu Lys Asp Tyr Pro Val
            20                  25                  30

ACT GTG GCC GTC AAT CTT CAG GAC GAG AAG CAC TGC AAG GCC TTG TGG    144
Thr Val Ala Val Asn Leu Gln Asp Glu Lys His Cys Lys Ala Leu Trp
        35                  40                  45

AGC CTC TTC CTA GCC CAG CGC TGG ATA GAG CAA CTG AAG ACT GTG GCA    192
Ser Leu Phe Leu Ala Gln Arg Trp Ile Glu Gln Leu Lys Thr Val Ala
    50                  55                  60

GGG TCT AAG ATG CAA ACG CTT CTG GAG GAC GTC AAC ACC GAG ATA CAT    240
Gly Ser Lys Met Gln Thr Leu Leu Glu Asp Val Asn Thr Glu Ile His
65                  70                  75                  80

TTT GTC ACC TCA TGT ACC TTC CAG CCC CTA CCA GAA TGT CTG CGA TTC    288
Phe Val Thr Ser Cys Thr Phe Gln Pro Leu Pro Glu Cys Leu Arg Phe
                85                  90                  95

GTA CAG ACC AAT ATA                                               303
Val Gln Thr Asn Ile
            100
```

(2) INFORMATION FOR SEQ ID NO:19:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 857 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:19:

```
GAAAGGGCTG TCACCCGGCT TGGCCCCTTC CACACCCAAC TGGGGCAAGC CTGACCCGGC   60

GACAGGAGGC ATGAGGGGCC CCCGGCCGAA ATG ACA GTG CTG GCG CCA GCC TGG   114
                                 Met Thr Val Leu Ala Pro Ala Trp
                                   1               5

AGC CCA ACA ACC TAT CTC CTC CTG CTG CTG CTG CTG AGC TCG GGA CTC    162
Ser Pro Thr Thr Tyr Leu Leu Leu Leu Leu Leu Leu Ser Ser Gly Leu
     10                  15                  20
```

```
AGT GGG ACC CAG GAC TGC TCC TTC CAA CAC AGC CCC ATC TCC TCC GAC   210
Ser Gly Thr Gln Asp Cys Ser Phe Gln His Ser Pro Ile Ser Ser Asp
 25                  30                  35                  40

TTC GCT GTC AAA ATC CGT GAG CTG TCT GAC TAC CTG CTT CAA GAT TAC   258
Phe Ala Val Lys Ile Arg Glu Leu Ser Asp Tyr Leu Leu Gln Asp Tyr
                 45                  50                  55

CCA GTC ACC GTG GCC TCC AAC CTG CAG GAC GAG GAG CTC TGC GGG GCG   306
Pro Val Thr Val Ala Ser Asn Leu Gln Asp Glu Glu Leu Cys Gly Ala
             60                  65                  70

CTC TGG CGG CTG GTC CTG GCA CAG CGC TGG ATG GAG CGG CTC AAG ACT   354
Leu Trp Arg Leu Val Leu Ala Gln Arg Trp Met Glu Arg Leu Lys Thr
         75                  80                  85

GTC GCT GGG TCC AAG ATG CAA GGC TTG CTG GAG CGC GTG AAC ACG GAG   402
Val Ala Gly Ser Lys Met Gln Gly Leu Leu Glu Arg Val Asn Thr Glu
     90                  95                  100

ATA CAC TTT GTC ACC AAA TGT GCC TTT CAG CCC CCC CCC AGC TGT CTT   450
Ile His Phe Val Thr Lys Cys Ala Phe Gln Pro Pro Pro Ser Cys Leu
105                 110                 115                 120

CGC TTC GTC CAG ACC AAC ATC TCC CGC CTC CTG CAG GAG ACC TCC GAG   498
Arg Phe Val Gln Thr Asn Ile Ser Arg Leu Leu Gln Glu Thr Ser Glu
                125                 130                 135

CAG CTG GTG GCG CTG AAG CCC TGG ATC ACT CGC CAG AAC TTC TCC CGG   546
Gln Leu Val Ala Leu Lys Pro Trp Ile Thr Arg Gln Asn Phe Ser Arg
            140                 145                 150

TGC CTG GAG CTG CAG TGT CAG CCC GAC TCC TCA ACC CTG CCA CCC CCA   594
Cys Leu Glu Leu Gln Cys Gln Pro Asp Ser Ser Thr Leu Pro Pro Pro
        155                 160                 165

TGG AGT CCC CGG CCC CTG GAG GCC ACA GCC CCG ACA GCC CCG CAG CCC   642
Trp Ser Pro Arg Pro Leu Glu Ala Thr Ala Pro Thr Ala Pro Gln Pro
    170                 175                 180

CCT CTG CTC CTC CTA CTG CTG CTG CCC GTG GGC CTC CTG CTG CTG GCC   690
Pro Leu Leu Leu Leu Leu Leu Leu Pro Val Gly Leu Leu Leu Leu Ala
185                 190                 195                 200

GCT GCC TGG TGC CTG CAC TGG CAG AGG ACG CGG CGG AGG ACA CCC CGC   738
Ala Ala Trp Cys Leu His Trp Gln Arg Thr Arg Arg Arg Thr Pro Arg
                205                 210                 215

CCT GGG GAG CAG GTG CCC CCC GTC CCC AGT CCC CAG GAC CTG CTG CTT   786
Pro Gly Glu Gln Val Pro Pro Val Pro Ser Pro Gln Asp Leu Leu Leu
            220                 225                 230

GTG GAG CAC TGACCTGGCC AAGGCCTCAT CCTGGGGAGG ATACGTAGGC          835
Val Glu His
        235

ACACAGAGGG GAGTCACCAG CC                                         857
```

(2) INFORMATION FOR SEQ ID NO:20:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 301 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:20:

```
GGT GCC CCC CGT CCC CAG TCC CCA GGA CCT GCT GCT TGT GGA GCA CTG     48
Gly Ala Pro Arg Pro Gln Ser Pro Gly Pro Ala Ala Cys Gly Ala Leu
  1               5                  10                  15

ACC TGG CCA AGG CCT CAT CCT GGG GAG GAT ACT GAG GCA CAC AGA GGG     96
Thr Trp Pro Arg Pro His Pro Gly Glu Asp Thr Glu Ala His Arg Gly
             20                  25                  30

GAG TCA CCA GCC AGA GGA TGC ATA GCC TGG ACA CAG AGG AAG TTG GCT    144
Glu Ser Pro Ala Arg Gly Cys Ile Ala Trp Thr Gln Arg Lys Leu Ala
         35                  40                  45

AGA GGC CGG TCC CTT CCT TGG GCC CCT CTC ATT CCC TCC CCA GAA TGG    192
Arg Gly Arg Ser Leu Pro Trp Ala Pro Leu Ile Pro Ser Pro Glu Trp
     50                  55                  60

AGG CAA CGC CAG AAT CCA GCA CCG GCC CCA TTT ACC CAA CTC TGT ACA    240
Arg Gln Arg Gln Asn Pro Ala Pro Ala Pro Phe Thr Gln Leu Cys Thr
 65                  70                  75                  80

AAG CCC TTG TCC CCA TGAAATTGTA TATAAATCAT CCTTTTCTAC CAAAAAAAAA    295
Lys Pro Leu Ser Pro
                 85

AAAAAA                                                             301
```

(2) INFORMATION FOR SEQ ID NO:21:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 1017 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:21:

```
GAATTCGCGG CCGCGTCGAG CCTGGCGGGA CTGAGCCCGA GACCTGCCCT CCTGTCACTT    60

CCAAGAACCT GTCACAGGCA TGAGGGGTCC CCGGCAGAG ATG ACA GTG CTG GCG      114
                                          Met Thr Val Leu Ala
                                            1               5

CCA GCC TGG AGC CCA AAT TCC TCC CTG TTG CTG CTG TTG CTG CTG CTG     162
Pro Ala Trp Ser Pro Asn Ser Ser Leu Leu Leu Leu Leu Leu Leu Leu
                    10                  15                  20

AGT CCT TGC CTG CGG GGG ACA CCT GAC TGT TAC TTC AGC CAC AGT CCC     210
Ser Pro Cys Leu Arg Gly Thr Pro Asp Cys Tyr Phe Ser His Ser Pro
                25                  30                  35

ATC TCC TCC AAC TTC AAA GTG AAG TTT AGA GAG TTG ACT GAC CAC CTG     258
Ile Ser Ser Asn Phe Lys Val Lys Phe Arg Glu Leu Thr Asp His Leu
            40                  45                  50

CTT AAA GAT TAC CCA GTC ACT GTG GCC GTC AAT CTT CAG GAC GAG AAG     306
Leu Lys Asp Tyr Pro Val Thr Val Ala Val Asn Leu Gln Asp Glu Lys
        55                  60                  65

CAC TGC AAG GCC TTG TGG AGC CTC TTC CTA GCC CAG CGC TGG ATA GAG     354
His Cys Lys Ala Leu Trp Ser Leu Phe Leu Ala Gln Arg Trp Ile Glu
    70                  75                  80                  85

CAA CTG AAG ACT GTG GCA GGG TCT AAG ATG CAA ACG CTT CTG GAG GAC     402
Gln Leu Lys Thr Val Ala Gly Ser Lys Met Gln Thr Leu Leu Glu Asp
                90                  95                  100

GTC AAC ACC GAG ATA CAT TTT GTC ACC TCA TGT ACC TTC CAG CCC CTA     450
Val Asn Thr Glu Ile His Phe Val Thr Ser Cys Thr Phe Gln Pro Leu
            105                 110                 115

CCA GAA TGT CTG CGA TTC GTC CAG ACC AAC ATC TCC CAC CTC CTG AAG     498
Pro Glu Cys Leu Arg Phe Val Gln Thr Asn Ile Ser His Leu Leu Lys
        120                 125                 130

GAC ACC TGC ACA CAG CTG CTT GCT CTG AAG CCC TGT ATC GGG AAG GCC     546
Asp Thr Cys Thr Gln Leu Leu Ala Leu Lys Pro Cys Ile Gly Lys Ala
    135                 140                 145

TGC CAG AAT TTC TCT CGG TGC CTG GAG GTG CAG TGC CAG CCG GAC TCC     594
Cys Gln Asn Phe Ser Arg Cys Leu Glu Val Gln Cys Gln Pro Asp Ser
150                 155                 160                 165

TCC ACC CTG CTG CCC CCA AGG AGT CCC ATA GCC CTA GAA GCC ACG GAG     642
Ser Thr Leu Leu Pro Pro Arg Ser Pro Ile Ala Leu Glu Ala Thr Glu
                170                 175                 180
```

```
CTC CCA GAG CCT CGG CCC AGG CAG CTG TTG CTC CTG CTG CTG CTG CTG     690
Leu Pro Glu Pro Arg Pro Arg Gln Leu Leu Leu Leu Leu Leu Leu Leu
            185                 190                 195

CTG CCT CTC ACA CTG GTG CTG CTG GCA GCC GCC TGG GGC CTT CGC TGG     738
Leu Pro Leu Thr Leu Val Leu Leu Ala Ala Ala Trp Gly Leu Arg Trp
        200                 205                 210

CAA AGG GCA AGA AGG AGG GGG GAG CTC CAC CCT GGG GTG CCC CTC CCC     786
Gln Arg Ala Arg Arg Arg Gly Glu Leu His Pro Gly Val Pro Leu Pro
    215                 220                 225

TCC CAT CCC TAGGATGCGA GCCTTGTGCA TCGTTGACTC AGCCAGGGTC              835
Ser His Pro
230

TTATCTCGAG TTGGGAACCA AAACAAGGAA CAAGCTAGGC AAGTGCTGTG CTGAGTTACA   895

TCCCCAGCCC AGAGGACACA CTGTCTGGGT ATGGCGATGG ACACTGTAAT TCCAGTGCTT   955

CTGGATTGGA CATGCTGAAA CTGGATACTG ACTTTAAGAA AAACAGAAAG GAAGAACCCC  1015
CC                                                                 1017
```

(2) INFORMATION FOR SEQ ID NO:22:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 57 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:22:

```
Gly Asn Gly Gly Pro Arg Ala Gln His His Gly Ala Thr Arg Leu Thr
1               5                   10                  15

Ala Thr Ala Leu Leu Thr Val Cys Pro Gly Leu Leu Leu Pro Leu Val
            20                  25                  30

Gly Thr Ser His Met Phe Phe Leu Pro Tyr Phe Leu Ser Phe Leu Ser
        35                  40                  45

Ser Phe Leu Lys Met Tyr Leu Tyr Val
    50                  55
```

(2) INFORMATION FOR SEQ ID NO:23:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 87 base pairs

(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:23:

```
GAT AGG GTC TCA TTA TTA TGC AGG CTA GGC CTG ACC CTG AAC TCA AAG      48
Asp Arg Val Ser Leu Leu Cys Arg Leu Gly Leu Thr Leu Asn Ser Lys
 1               5                  10                  15

CAA TCC TCC TGC CTC AGT GTC CTG AGT GCT GGG ATT ACA                  87
Gln Ser Ser Cys Leu Ser Val Leu Ser Ala Gly Ile Thr
            20                  25
```

(2) INFORMATION FOR SEQ ID NO:24:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 344 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:24:

```
GGTAACGGTG GCCCCAGAGC CCAGCACCAT GGTGCCACCA GGCTCACAGC CACAGCCTTG   60

CTAACTGTGT GTCCAGGGCT TCTGCTCCCA CTAGTTGGCA CTTCACACAT GTTCTTTCTC  120

CCTTATTTTC TCTCTTTTCT TTCTTCTTTT TTAAAGATGT ATCTTTATGT GTGAGTGTTT  180

TACCTACATG CCTGTAAGTG CACTGAATGT GTGTCTGGTG CCTGCAGAGG CCAGAAGAGG  240

GCACCAGATC CCCTGAAACT GGAGTCTCTN NGCTCCGTGT GAACCACCAC GTGGTGCTGG  300

GACCCAGGTC CAATGCAAGA GCACCCAGGG TTCTTACCTG CTGA                    344
```

(2) INFORMATION FOR SEQ ID NO:25:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 18 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:25:

TTYGTNCARA CNAAYATH 18

(2) INFORMATION FOR SEQ ID NO:26:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 18 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:26:

TTYGTNCARA CNTGYATH 18

(2) INFORMATION FOR SEQ ID NO:27:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 18 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:27:

TTYGTNCARA CNAGYATH 18

(2) INFORMATION FOR SEQ ID NO:28:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 18 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:28:

TTYGTNCARA CNTCNATH 18

(2) INFORMATION FOR SEQ ID NO:29:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 18 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:29:

TTYGTNCARA CNACNATH 18

(2) INFORMATION FOR SEQ ID NO:30:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 18 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:30:

GAYTAYCCNG TNACNGTN 18

(2) INFORMATION FOR SEQ ID NO:31:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 18 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:31:

ACNCCNGAYA TNTAYTTY
18

(2) INFORMATION FOR SEQ ID NO:32:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 18 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:32:

TGGATHGARC ARCTNAAR 18

(2) INFORMATION FOR SEQ ID NO:33:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 18 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:33:

TGGATHGARC ARTTRAAR 18

(2) INFORMATION FOR SEQ ID NO:34:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 18 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:34:

AAYTTYAARG TNAARTTY 18

(2) INFORMATION FOR SEQ ID NO:35:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 87 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:35:

ACTCCTGACT GTTACTTCAG CCACAGTCCC ATCTCCTCCA ACTTCAAAGT GAAGTTTAGA 60

GAGTTGACTG ACCACCTGCT TAAAGAT 87

(2) INFORMATION FOR SEQ ID NO:36:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 94 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:36:

GGATCCCGGG TACCTTCTAG AATTCCGGAG CGGCCGCTGC AGATCTCATC ACCATCACCA 60

TCACGATTAC AAGGACGATG ACGATAAGTA ATGA 94

(2) INFORMATION FOR SEQ ID NO:37:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 29 amino acids
(B) TYPE: amino acid

(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:37:

```
Asp Pro Gly Tyr Leu Leu Glu Phe Arg Ser Gly Arg Cys Arg Ser His
1               5                  10                  15

His His His His His Asp Tyr Lys Asp Asp Asp Asp Lys
            20                  25
```

WHAT IS CLAIMED IS:

1. A substantially pure mammalian Flt3 ligand or a fragment thereof.

2. An isolated nucleic acid encoding a mammalian Flt3 ligand or a fragment thereof.

3. A recombinant vector comprising a nucleic acid of claim 2.

4. A host cell comprising a recombinant vector of claim 3.

5. A method for making a mammalian Flt3 ligand or a fragment thereof comprising culturing a host cell of claim 4 under conditions in which the nucleic acid is expressed.

6. An antibody or binding fragment thereof which specifically binds to a Flt3 ligand or fragment of claim 1.

7. A pharmaceutical composition for modifying the biological activity of cells bearing Flt3 ligand receptors comprising a physiologically acceptable carrier and a mammalian Flt3 ligand or an antibody or binding fragment thereof which specifically binds to such ligand.

8. A method for the manufacture of a pharmaceutical composition for modifying the biological activity of cells bearing Flt3 ligand receptors comprising admixing a physiologically acceptable carrier and a mammalian Flt3 ligand or an antibody or binding fragment thereof which specifically binds to such ligand.

9. A kit comprising in separate containers:

(a) a substantially pure mammalian Flt3 ligand or a fragment thereof;

(b) an antibody or binding fragment thereof which specifically binds to a mammalian Flt3 ligand or fragment thereof; or

(c) a nucleic acid encoding a mammalian Flt3 ligand or fragment thereof.

10. The ligand or fragment thereof, nucleic acid, method, antibody, pharmaceutical composition or kit of any one of claims 1 to 9 in which the Flt3 ligand is a mouse or human Flt3 ligand.

11. The ligand or fragment thereof, nucleic acid, method, antibody, pharmaceutical composition or kit of claim 10 in which the sequence of the Flt3 ligand, fragment thereof or nucleic acid is defined by any one of SEQ ID NOs: 1 to 24.

PCT WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 5 :
G01N 1/34, 33/48, C12N 5/00, 1/21, A61K 37/02, 48/00, C07K 13/00, 15/28, C07H 21/04, C12N 15/12, 15/64, A01K 67/00

A1

(11) International Publication Number: **WO 94/28391**

(43) International Publication Date: 8 December 1994 (08.12.94)

(21) International Application Number: PCT/US94/05365

(22) International Filing Date: 12 May 1994 (12.05.94)

(30) Priority Data:

08/068,394	24 May 1993 (24.05.93)	US
08/106,463	12 August 1993 (12.08.93)	US
08/111,758	25 August 1993 (25.08.93)	US
08/162,407	3 December 1993 (03.12.93)	US
08/209,502	7 March 1994 (07.03.94)	US

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(81) Designated States: AT, AU, BB, BG, BR, BY, CA, CH, CN, CZ, DE, DK, ES, FI, GB, HU, JP, KP, KR, KZ, LK, LU, LV, MD, MN, MW, NL, NO, NZ, PL, PT, RO, RU, SD, SE, SK, UA, UZ, VN, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).

Published
With international search report.

(54) Title: **LIGANDS FOR FLT3 RECEPTORS**

(57) Abstract

Ligands for flt3 receptors capable of transducing self-renewal signals to regulate the growth, proliferation or differentiation of progenitor cells and stem cells are disclosed. The invention is directed to flt3-L as an isolated protein, the DNA encoding the flt3-L, host cells transfected with cDNAs encoding flt3-L, compositions comprising flt3-L, methods of improving gene transfer to a mammal using flt3-L, and methods of improving transplantations using flt3-L. Flt3-L finds use in treating patients with anemia, AIDS and various cancers.

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TITLE

LIGANDS FOR FLT3 RECEPTORS

CROSS-REFERENCE TO RELATED APPLICATIONS

This is a continuation-in-part of United States Application 08/209,502 filed March 7, 1994, which is a continuation-in-part of United States Application 08/162,407, filed December 3, 1993, which is a continuation-in-part of United States Application 08/111,758, filed August 25, 1993, which is a continuation-in-part of United States Application 08/106,463, filed August 12, 1993, which in turn is a continuation-in-part of United States Application 08/068,394, filed May 24, 1993, abandoned.

FIELD OF THE INVENTION

The present invention relates to mammalian flt3-ligands, the nucleic acids encoding such ligands, processes for production of recombinant flt3-ligands, pharmaceutical compositions containing such ligands, and their use in various therapies.

BACKGROUND OF THE INVENTION

Blood cells originate from hematopoietic stem cells that become committed to differentiate along certain lineages, i.e., erythroid, megakaryocytic, granulocytic, monocytic, and lymphocytic. Cytokines that stimulate the proliferation and maturation of cell precursors are called colony stimulating factors ("CSFs"). Several CSFs are produced by T-lymphocytes, including interleukin-3 ("IL-3"), granulocyte-monocyte CSF (GM-CSF), granulocyte CSF (G-CSF), and monocyte CSF (M-CSF). These CSFs affect both mature cells and stem cells. Heretofore no factors have been discovered that are able to predominantly affect stem cells.

Tyrosine kinase receptors ("TKRs") are growth factor receptors that regulate the proliferation and differentiation of a number of cells (Yarden, Y. & Ullrich, A. *Annu. Rev. Biochem.*, 57, 443-478, 1988; and Cadena, D.L. & Gill, G.N. *FASEB J.*, 6, 2332-2337, 1992). Certain TKRs function within the hematopoietic system. For example, signaling through the colony-stimulating factor type 1 ("CSF-1"), receptor c-fms regulates the survival, growth and differentiation of monocytes (Stanley *et al.*, *J.*

Cell Biochem., 21, 151-159, 1983). Steel factor ("SF", also known as mast cell growth factor, stem cell factor or kit ligand), acting through c-kit, stimulates the proliferation of cells in both myeloid and lymphoid compartments.

Flt3 (Rosnet et al. *Oncogene*, 6, 1641-1650, 1991) and flk-2 (Matthews et al., *Cell*, 65, 1143-1152, 1991) are variant forms of a TKR that is related to the c-fms and c-kit receptors. The flk-2 gene product is expressed on hematopoietic and progenitor cells, while the flt3 gene product has a more general tissue distribution. The flt3 and flk-2 receptor proteins are similar in amino acid sequence and vary at two amino acid residues in the extracellular domain and diverge in a 31 amino acid segment located near the C-termini (Lyman et al., *Oncogene*, 8, 815-822, 1993).

Flt3-ligand ("flt3-L") has been found to regulate the growth and differentiation of progenitor and stem cells and is likely to possess clinical utility in treating hematopoietic disorders, in particular, aplastic anemia and myelodysplastic syndromes. Additionally, flt3-L will be useful in allogeneic, syngeneic or autologous bone marrow transplants in patients undergoing cytoreductive therapies, as well as cell expansion. Flt3-L will also be useful in gene therapy and progenitor and stem cell mobilization systems.

Cancer is treated with cytoreductive therapies that involve administration of ionizing radiation or chemical toxins that kill rapidly dividing cells. Side effects typically result from cytotoxic effects upon normal cells and can limit the use of cytoreductive therapies. A frequent side effect is myelosuppression, or damage to bone marrow cells that give rise to white and red blood cells and platelets. As a result of myelosuppression, patients develop cytopenia, or blood cell deficits, that increase risk of infection and bleeding disorders.

Cytopenias increase morbidity, mortality, and lead to under-dosing in cancer treatment. Many clinical investigators have manipulated cytoreductive therapy dosing regimens and schedules to increase dosing for cancer therapy, while limiting damage to bone marrow. One approach involves bone marrow or peripheral blood cell transplants in which bone marrow or circulating hematopoietic progenitor or stem cells are removed before cytoreductive therapy and then reinfused following therapy to restore hematopoietic function. U.S. Patent No. 5,199,942, incorporated herein by reference, describes a method for using GM-CSF, IL-3, SF, GM-CSF/IL-3 fusion proteins,

erythropoietin ("EPO") and combinations thereof in autologous transplantation regimens.

High-dose chemotherapy is therapeutically beneficial because it can produce an increased frequency of objective response in patients with metastatic cancers, particularly breast cancer, when compared to standard dose therapy. This can result in extended disease-free remission for some even poor-prognosis patients. Nevertheless, high-dose chemotherapy is toxic and many resulting clinical complications are related to infections, bleeding disorders and other effects associated with prolonged periods of myelosuppression.

Myelodysplastic syndromes are stem cell disorders characterized by impaired cellular maturation, progressive pancytopenia, and functional abnormalities of mature cells. They have also been characterized by variable degrees of cytopenia, ineffective erythropoiesis and myelopoiesis with bone marrow cells that are normal or increased in number and that have peculiar morphology. Bennett et. al. (*Br. J. Haematol.* 1982; 51:189-199) divided these disorders into five subtypes: refractory anemia, refractory anemia with ringed sideroblasts, refractory anemia with excess blasts, refractory anemia with excess blasts in transformation, and chronic myelomonocytic leukemia. Although a significant percentage of these patients develop acute leukemia, a majority die from infectious or hemorrhagic complications. Treatment of theses syndromes with retinoids, vitamin D, and cytarabine has not been successful. Most of the patients suffering from these syndromes are elderly and are not suitable candidates for bone marrow transplantation or aggressive antileukemic chemotherapy.

Aplastic anemia is another disease entity that is characterized by bone marrow failure and severe pancytopenia. Unlike myelodysplastic syndrome, the bone marrow is acellular or hypocellular in this disorder. Current treatments include bone marrow transplantation from a histocompatible donor or immunosuppressive treatment with antithymocyte globulin (ATG). Similarly to myelodysplastic syndrome, most patients suffering from this syndrome are elderly and are unsuitable for bone marrow transplantation or for aggressive antileukemic chemotherapy. Mortality in these patients is exceedingly high from infectious or hemorrhagic complications.

Anemia is common in patients with acquired immune deficiency syndrome (AIDS). The anemia is usually more severe in patients receiving zidovudine therapy. Many important retroviral agents, anti-infectives, and anti-neoplastics suppress

erythropoiesis. Recombinant EPO has been shown to normalize the patient's hematocrit and hemaoglobin levels, however, usually very high doses are required. A growth factor that stimulates proliferation of the erythroid lineage could be used alone or in combination with EPO or other growth factors to treat such patients and reduce the number of transfusions required. A growth factor that could also increase the number of T cells would find particular use in treating AIDS patients.

SUMMARY OF THE INVENTION

The present invention pertains to biologically active flt3-ligand (flt3-L) as an isolated or homogeneous protein. In addition, the invention is directed to isolated DNAs encoding flt3-L and to expression vectors comprising a cDNA encoding flt3-L. Within the scope of this invention are host cells that have been transfected or transformed with expression vectors that comprise a cDNA encoding flt3-L, and processes for producing flt3-L by culturing such host cells under conditions conducive to expression of flt3-L.

Flt3-L can be used to prepare pharmaceutical compositions to be used in allogeneic, syngeneic or autologous transplantation methods. Pharmaceutical compositions may comprise flt3-L alone or in combination with other growth factors, such as interleukins, colony stimulating factors, protein tyrosine kinases and cytokines.

The invention includes methods of using flt3-L compositions in gene therapy and in treatment of patients suffering from myelodysplastic syndrome, aplastic anemia, HIV infection (AIDS) and cancers, such as breast cancer, lymphoma, small cell lung cancer, multiple myeloma, neuroblastoma, acute leukemia, testicular tumors, and ovarian cancer.

The present invention also pertains to antibodies, and in particular monoclonal antibodies, that are immunoreactive with flt3-L. Fusion proteins comprising a soluble portion of flt3-L and the constant domain of an immunoglobulin protein are also embodied in the invention.

The present invention also is directed to the use of flt3-L in peripheral blood progenitor or stem cell transplanation procedures. Typically, peripheral blood progenitor cells or stem cells are removed from a patient prior to myelosuppressive cytoreductive therapy, and then readministered to the patient concurrent with or

following cytoreductive therapy to counteract the myelosuppressive effects of such therapy. The present invention provides for the use of an effective amount of flt3-L in at least one of the following manners: (i) flt3-L is administered to the patient prior to collection of the progenitor or stem cells to increase or mobilize the numbers of such circulating cells; (ii) following collection of the patient's progenitor or stem cells, flt3-L is used to expand such cells *ex vivo*; and (iii) flt3-L is administered to the patient following transplantation of the collected progenitor or stem cells to facilitate engraftment thereof. The transplantation method of the invention can further comprise the use of an effective amount of a cytokine in sequential or concurrent combination with the flt3-L. Such cytokines include, but are not limited to interleukins ("IL") IL-1, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-11, IL-12, IL-13, IL-14 or IL-15, a CSF selected from the group consisting of G-CSF, GM-CSF, M-CSF, or GM-CSF/IL-3 fusions, or other growth factors such as CSF-1, SF, EPO, leukemia inhibitory factor ("LIF") or fibroblast growth factor ("FGF"). The flt3-L is also useful in the same way for syngeneic or allogeneic transplantations.

The invention further includes a progenitor or stem cell expansion media comprising cell growth media, autologous serum, and flt3-L alone or in combination with a cytokine from the group listed above.

The invention further includes the use of flt3-L to expand progenitor or stem cells collected from umbilical cord blood. The expansion may be performed with flt3-L alone or in sequential or concurrent combination with a cytokine from the group listed above.

The invention further includes the use of flt3-L in gene therapy. Flt3-L permits proliferation and culturing of the early hematopoietic progenitor or stem cells that are to be transfected with exogenous DNA for use in gene therapy. Alternatively, a cDNA encoding flt3-L may be transfected into cells in order to ultimately deliver its gene product to the targeted cell or tissue.

In addition, the invention includes the use of flt3-L to stimulate production of erythroid cells *in vivo* for the treatment of anemia. Such use comprises administering flt3-L to the patient in need of such erythroid cell stimulation in conjunction with or following cytoreductive therapy. The treatment can include co-administration of another growth factor selected from the cytokines from the group listed above. Preferred cytokines for use in this treatment include EPO, IL-3, G-CSF and GM-CSF.

Such treatment is especially useful for AIDS patients, and preferably for AIDS patients receiving AZT therapy.

Since flt3-L stimulates the production of stem cells, other non-hematopoietic stem cells bearing flt3 receptors can be affected by the flt3-L of the invention. Flt3-L is useful in *in vitro* fertilization procedures and can be used *in vivo* in the treatment of infertility conditions. In the gut, the flt3 ligand is useful in treating intestinal damage resulting from irradiation or chemotherapy. The flt3-L can be also used to treat patients infected with the human immunodeficiency virus (HIV). Such treatment would encompass the administration of the flt3-L to stimulate *in vivo* production, as well as the *ex vivo* expansion, of T cells and erythroid cells. Such treatment can prevent the deficiency of T cells, in particular CD4-positive T cells, and may elevate the patient's immune reponse against the virus, thereby improving the quality of life of the patient. The flt3-L can be used to stimulate the stem cells that lead to the development of hair follicles, thereby promoting hair growth.

In addition, flt3-L can be bound to a solid phase matrix and used to affinity-purify or separate cells that express flt3 on their cell surface. The invention encompasses separating cells having the flt3 receptor on the surface thereof from a mixture of cells in solution, comprising contacting the cells in the mixture with a contacting surface having a flt3-binding molecule thereon, and separating the contacting surface and the solution. Once separated, the cells can be expanded *ex vivo* using flt3-L and administered to a patient.

DETAILED DESCRIPTION OF THE INVENTION

A cDNA encoding murine flt3-L has been isolated and is disclosed in SEQ ID NO:1. A cDNA encoding human flt3-L also has been isolated and is disclosed in SEQ ID NO:5. This discovery of cDNAs encoding murine and human flt3-L enables construction of expression vectors comprising cDNAs encoding flt3-L; host cells transfected or transformed with the expression vectors; biologically active murine and human flt3-L as homogeneous proteins; and antibodies immunoreactive with the murine and the human flt3-L.

Flt3-L is useful in the enhancement, stimulation, proliferation or growth of cells expressing the flt3 receptor, including non-hematopoietic cells. Since the flt3 receptor is found in the brain, placenta, and tissues of nervous and hematopoietic origin, and

finds distribution in the testis, ovaries, lymph node, spleen, thymus and fetal liver, treatment of a variety of conditions associated with tissue damage thereof is possible. While not limited to such, particular uses of the flt3-L are described infra.

As used herein, the term "flt3-L" refers to a genus of polypeptides that bind and complex independently with flt3 receptor found on progenitor and stem cells. The term "flt3-L" encompasses proteins having the amino acid sequence 1 to 231 of SEQ ID NO:2 or the amino acid sequence 1 to 235 of SEQ ID NO:6, as well as those proteins having a high degree of similarity or a high degree of identity with the amino acid sequence 1 to 231 of SEQ ID NO:2 or the amino acid sequence 1 to 235 of SEQ ID NO:6, and which proteins are biologically active and bind the flt3 receptor. In addition, the term refers to biologically active gene products of the DNA of SEQ ID NO:1 or SEQ ID NO:5. Further encompassed by the term "flt3-L" are the membrane-bound proteins (which include an intracellular region, a membrane region, and an extracellular region), and soluble or truncated proteins which comprise primarily the extracellular portion of the protein, retain biological activity and are capable of being secreted. Specific examples of such soluble proteins are those comprising the sequence of amino acids 28-163 of SEQ ID NO:2 and amino acids 28-160 of SEQ ID NO:6.

The term "biologically active" as it refers to flt3-L, means that the flt3-L is capable of binding to flt3. Alternatively, "biologically active" means the flt3-L is capable of transducing a stimulatory signal to the cell through the membrane-bound flt3.

"Isolated" means that flt3-L is free of association with other proteins or polypeptides, for example, as a purification product of recombinant host cell culture or as a purified extract.

A "flt3-L variant" as referred to herein, means a polypeptide substantially homologous to native flt3-L, but which has an amino acid sequence different from that of native flt3-L (human, murine or other mammalian species) because of one or more deletions, insertions or substitutions. The variant amino acid sequence preferably is at least 80% identical to a native flt3-L amino acid sequence, most preferably at least 90% identical. The percent identity may be determined, for example, by comparing sequence information using the GAP computer program, version 6.0 described by Devereux et al. (*Nucl. Acids Res.* 12:387, 1984) and available from the University of Wisconsin Genetics Computer Group (UWGCG). The GAP program utilizes the

alignment method of Needleman and Wunsch (*J. Mol. Biol.* 48:443, 1970), as revised by Smith and Waterman (*Adv. Appl. Math* 2:482, 1981). The preferred default parameters for the GAP program include: (1) a unary comparison matrix (containing a value of 1 for identities and 0 for non-identities) for nucleotides, and the weighted comparison matrix of Gribskov and Burgess, *Nucl. Acids Res.* 14:6745, 1986, as described by Schwartz and Dayhoff, eds., *Atlas of Protein Sequence and Structure*, National Biomedical Research Foundation, pp. 353-358, 1979; (2) a penalty of 3.0 for each gap and an additional 0.10 penalty for each symbol in each gap; and (3) no penalty for end gaps. Variants may comprise conservatively substituted sequences, meaning that a given amino acid residue is replaced by a residue having similar physiochemical characteristics. Examples of conservative substitutions include substitution of one aliphatic residue for another, such as Ile, Val, Leu, or Ala for one another, or substitutions of one polar residue for another, such as between Lys and Arg; Glu and Asp; or Gln and Asn. Other such conservative substitutions, for example, substitutions of entire regions having similar hydrophobicity characteristics, are well known. Naturally occurring flt3-L variants are also encompassed by the invention. Examples of such variants are proteins that result from alternate mRNA splicing events or from proteolytic cleavage of the flt3-L protein, wherein the flt3-L binding property is retained. Alternate splicing of mRNA may yield a truncated but biologically active flt3-L protein, such as a naturally occurring soluble form of the protein, for example. Variations attributable to proteolysis include, for example, differences in the N- or C-termini upon expression in different types of host cells, due to proteolytic removal of one or more terminal amino acids from the flt3-L protein (generally from 1-5 terminal amino acids).

The term "autologous transplantation" is described in U.S. Patent No. 5,199,942, which is incorporated herein by reference. Briefly, the term means a method for conducting autologous hematopoietic progenitor or stem cell transplantation, comprising: (1) collecting hematopoietic progenitor cells or stem cells from a patient prior to cytoreductive therapy; (2) expanding the hematopoietic progenitor cells or stem cells *ex vivo* with flt3-L to provide a cellular preparation comprising increased numbers of hematopoietic progenitor cells or stem cells; and (3) administering the cellular preparation to the patient in conjunction with or following cytoreductive therapy. Progenitor and stem cells may be obtained from peripheral blood harvest or bone marrow explants. Optionally, one or more cytokines, selected from the group listed above can be combined with flt3-L to aid in the proliferation of particular hematopoietic cell types or affect the cellular function of the resulting proliferated

hematopoietic cell population. Of the foregoing, SF, IL-1, IL-3, EPO, G-CSF, GM-CSF and GM-CSF/IL-3 fusions are preferred, with G-CSF, GM-CSF and GM-CSF/IL-3 fusions being especially preferred. The term "allogeneic transplantation" means a method in which bone marrow or peripheral blood progenitor cells or stem cells are removed from a mammal and administered to a different mammal of the same species. The term "syngeneic transplantation" means the bone marrow transplantation between gentically identical mammals.

The transplantation method of the invention described above optionally comprises a preliminary *in vivo* procedure comprising administering flt3-L alone or in sequential or concurrent combination with a recruitment growth factor to a patient to recruit the hematopoietic cells into peripheral blood prior to their harvest. Suitable recruitment factors are listed above, and preferred recruitment factors are flt3-L, SF, IL-1 and IL-3.

The method of the invention described above optionally comprises a subsequent *in vivo* procedure comprising administering flt3-L alone or in sequential or concurrent combination with an engraftment growth factor to a patient following transplantation of the cellular preparation to facilitate engraftment and augment proliferation of engrafted hematopoietic progenitor or stem cells from the cellular preparation. Suitable engraftment factors are listed above, and the preferred engraftment factors are GM-CSF, G-CSF, IL-3, IL-1, EPO and GM-CSF/IL-3 fusions.

The invention further includes a progenitor or stem cell expansion media comprising cell growth media, autologous serum, and flt3-L alone or in combination with a cytokine growth factor from the list above. Preferred growth factors are SF, GM-CSF, IL-3, IL-1, G-CSF, EPO, and GM-CSF/IL-3 fusions.

In particular, flt3-L can be used to stimulate the proliferation of hematopoietic and non-hematopoietic stem cells. Such stimulation is beneficial when specific tissue damage has occurred to these tissues. As such, flt3-L may be useful in treating neurological damage and may be a growth factor for nerve cells. It is probable that flt3-L would be useful in *in vitro* fertilization procedures and likely can be used *in vivo* in the treatment of infertility conditions. Flt3-L would be useful in treating intestinal damage resulting from irradiation or chemotherapy. Since the flt3 receptor is distributed on stem cells leading to the development of hair follicles, flt3-L would likely be useful to promote hair growth.

Since flt3-L has been shown to stimulate T cell proliferation as well as erythrocytes (see Examples, infra), flt3-L finds use in the treatment of patients infected with the human immunodeficiency virus (HIV). Such treatment would encompass the administration of flt3-L to stimulate *in vivo* production, as well as the *ex vivo* expansion, of T cells. In addition, flt3-L can prevent the deficiency of $CD4^+$ T cells. Such treatment may elevate or maintain a patient's immune reponse against the virus, thereby improving or maintaining a patient's quality of life. In addition, such *in vivo* treatment would stimulate cells of the erythroid lineage, thereby improving a patient's hematocrit and hemaglobin levels. Flt3-L can be administered in this setting alone or in sequential or concurrent combination with cytokines selected from the group listed above.

Flt3-L is useful in gene therapy due to its specificity for progenitor and stem cells. Gene therapy involves administration of exogenous DNA-transfected cells to a host that are allowed to engraft. See e.g., Boggs, *International J. Cell Cloning*, 8:80-96, (1990); Kohn et. al., *Cancer Invest.*, 7(2):179-192 (1989); Lehn, *Bone Marrow Transpl..*, 5:287-293 (1990); and Verma, *Scientific American*, pp. 68-84 (1990). Using gene therapy methods known in the art, a method of transferring a gene to a mammal comprises the steps of (a) culturing early hematopoietic cells in media comprising flt3-L alone or in sequential or concurrent combination with a cyokine selected from the group listed above; (b) transfecting the cultured cells from step (a) with the exogenous gene; and (c) administering the transfected cells to the mammal. Within this method is the novel method of transfecting progenitor or stem cells with a gene comprising the steps of: (a) and (b) above. Furthermore, using the same or simolar methods, the cDNA encoding the flt3-L can be transfected into such delivery cells to deliver the flt3-L gene product to the targetted tissue.

Example 1 describes the construction of a novel flt3:Fc fusion protein utilized in the screening for flt3-L. Other antibody Fc regions may be substituted for the human IgG1 Fc region described in Example 1. Other suitable Fc regions are those that can bind with high affinity to protein A or protein G, and include the Fc region of human IgG1 or fragments of the human or murine IgG1 Fc region, e.g., fragments comprising at least the hinge region so that interchain disulfide bonds will form. The flt3:Fc fusion protein offers the advantage of being easily purified. In addition, disulfide bonds form between the Fc regions of two separate fusion protein chains, creating dimers. The dimeric flt3:Fc receptor was chosen for the potential advantage of higher affinity

binding of flt3-L, in view of the possibility that the ligand being sought would be multimeric.

As described supra., an aspect of the invention is soluble flt3-L polypeptides. Soluble flt3-L polypeptides comprise all or part of the extracellular domain of a native flt3-L but lack the transmembrane region that would cause retention of the polypeptide on a cell membrane. Soluble flt3-L polypeptides advantageously comprise the native (or a heterologous) signal peptide when initially synthesized to promote secretion, but the signal peptide is cleaved upon secretion of flt3-L from the cell. Soluble flt3-L polypeptides encompassed by the invention retain the ability to bind the flt3 receptor. Indeed, soluble flt3-L may also include part of the transmembrane region or part of the cytoplasmic domain or other sequences, provided that the soluble flt3-L protein can be secreted.

Soluble flt3-L may be identified (and distinguished from its non-soluble membrane-bound counterparts) by separating intact cells which express the desired protein from the culture medium, e.g., by centrifugation, and assaying the medium (supernatant) for the presence of the desired protein. The presence of flt3-L in the medium indicates that the protein was secreted from the cells and thus is a soluble form of the desired protein.

Soluble forms of flt3-L possess many advantages over the native bound flt3-L protein. Purification of the proteins from recombinant host cells is feasible, since the soluble proteins are secreted from the cells. Further, soluble proteins are generally more suitable for intravenous administration.

Examples of soluble flt3-L polypeptides include those comprising a substantial portion of the extracellular domain of a native flt3-L protein. Such soluble mammalian flt3-L proteins comprise amino acids 28 through 188 of SEQ ID NO:2 or amino acids 28 through 182 of SEQ ID NO:6. In addition, truncated soluble flt3-L proteins comprising less than the entire extracellular domain are included in the invention. Such truncated soluble proteins are represented by the sequence of amino acids 28-163 of SEQ ID NO:2, and amino acids 28-160 of SEQ ID NO:6. When initially expressed within a host cell, soluble flt3-L may additionally comprise one of the heterologous signal peptides described below that is functional within the host cells employed. Alternatively, the protein may comprise the native signal peptide, such that the mammalian flt3-L comprises amino acids 1 through 188 of SEQ ID NO:2 or amino

acids 1 through 182 of SEQ ID NO:6. In one embodiment of the invention, soluble flt3-L was expressed as a fusion protein comprising (from N- to C-terminus) the yeast α factor signal peptide, a FLAG® peptide described below and in U.S. Patent No. 5,011,912, and soluble flt3-L consisting of amino acids 28 to 188 of SEQ ID NO:2. This recombinant fusion protein is expressed in and secreted from yeast cells. The FLAG® peptide facilitates purification of the protein, and subsequently may be cleaved from the soluble flt3-L using bovine mucosal enterokinase. Isolated DNA sequences encoding soluble flt3-L proteins are encompassed by the invention.

Truncated flt3-L, including soluble polypeptides, may be prepared by any of a number of conventional techniques. A desired DNA sequence may be chemically synthesized using techniques known per se. DNA fragments also may be produced by restriction endonuclease digestion of a full length cloned DNA sequence, and isolated by electrophoresis on agarose gels. Linkers containing restriction endonuclease cleavage site(s) may be employed to insert the desired DNA fragment into an expression vector, or the fragment may be digested at cleavage sites naturally present therein. The well known polymerase chain reaction procedure also may be employed to amplify a DNA sequence encoding a desired protein fragment. As a further alternative, known mutagenesis techniques may be employed to insert a stop codon at a desired point, e.g., immediately downstream of the codon for the last amino acid of the extracellular domain.

In another approach, enzymatic treatment (e.g., using *Bal* 31 exonuclease) may be employed to delete terminal nucleotides from a DNA fragment to obtain a fragment having a particular desired terminus. Among the commercially available linkers are those that can be ligated to the blunt ends produced by *Bal* 31 digestion, and which contain restriction endonuclease cleavage site(s). Alternatively, oligonucleotides that reconstruct the N- or C-terminus of a DNA fragment to a desired point may be synthesized and ligated to the DNA fragment. The synthesized oligonucleotide may contain a restriction endonuclease cleavage site upstream of the desired coding sequence and position an initiation codon (ATG) at the N-terminus of the coding sequence.

As stated above, the invention provides isolated or homogeneous flt3-L polypeptides, both recombinant and non-recombinant. Variants and derivatives of native flt3-L proteins that retain the desired biological activity (e.g., the ability to bind flt3) may be obtained by mutations of nucleotide sequences coding for native flt3-L polypeptides. Alterations of the native amino acid sequence may be accomplished by

any of a number of conventional methods. Mutations can be introduced at particular loci by synthesizing oligonucleotides containing a mutant sequence, flanked by restriction sites enabling ligation to fragments of the native sequence. Following ligation, the resulting reconstructed sequence encodes an analog having the desired amino acid insertion, substitution, or deletion.

Alternatively, oligonucleotide-directed site-specific mutagenesis procedures can be employed to provide an altered gene wherein predetermined codons can be altered by substitution, deletion or insertion. Exemplary methods of making the alterations set forth above are disclosed by Walder et al. (*Gene* 42:133, 1986); Bauer et al. (*Gene* 37:73, 1985); Craik (*BioTechniques*, January 1985, 12-19); Smith et al. (*Genetic Engineering: Principles and Methods*, Plenum Press, 1981); Kunkel (*Proc. Natl. Acad. Sci. USA* 82:488, 1985); Kunkel et al. (*Methods in Enzymol.* 154:367, 1987); and U.S. Patent Nos. 4,518,584 and 4,737,462 all of which are incorporated by reference.

Flt3-L may be modified to create flt3-L derivatives by forming covalent or aggregative conjugates with other chemical moieties, such as glycosyl groups, lipids, phosphate, acetyl groups and the like. Covalent derivatives of flt3-L may be prepared by linking the chemical moieties to functional groups on flt3-L amino acid side chains or at the N-terminus or C-terminus of a flt3-L polypeptide or the extracellular domain thereof. Other derivatives of flt3-L within the scope of this invention include covalent or aggregative conjugates of flt3-L or its fragments with other proteins or polypeptides, such as by synthesis in recombinant culture as N-terminal or C-terminal fusions. For example, the conjugate may comprise a signal or leader polypeptide sequence (e.g. the α-factor leader of *Saccharomyces*) at the N-terminus of a flt3-L polypeptide. The signal or leader peptide co-translationally or post-translationally directs transfer of the conjugate from its site of synthesis to a site inside or outside of the cell membrane or cell wall.

Flt3-L polypeptide fusions can comprise peptides added to facilitate purification and identification of flt3-L. Such peptides include, for example, poly-His or the antigenic identification peptides described in U.S. Patent No. 5,011,912 and in Hopp et al., *Bio/Technology* 6:1204, 1988.

The invention further includes flt3-L polypeptides with or without associated native-pattern glycosylation. Flt3-L expressed in yeast or mammalian expression systems (e.g., COS-7 cells) may be similar to or significantly different from a native

flt3-L polypeptide in molecular weight and glycosylation pattern, depending upon the choice of expression system. Expression of flt3-L polypeptides in bacterial expression systems, such as *E. coli*, provides non-glycosylated molecules.

Equivalent DNA constructs that encode various additions or substitutions of amino acid residues or sequences, or deletions of terminal or internal residues or sequences not needed for biological activity or binding are encompassed by the invention. For example, N-glycosylation sites in the flt3-L extracellular domain can be modified to preclude glycosylation, allowing expression of a reduced carbohydrate analog in mammalian and yeast expression systems. N-glycosylation sites in eukaryotic polypeptides are characterized by an amino acid triplet Asn-X-Y, wherein X is any amino acid except Pro and Y is Ser or Thr. The murine and human flt3-L proteins each comprise two such triplets, at amino acids 127-129 and 152-154 of SEQ ID NO:2, and at amino acids 126-128 and 150-152 of SEQ ID NO:6, respectively. Appropriate substitutions, additions or deletions to the nucleotide sequence encoding these triplets will result in prevention of attachment of carbohydrate residues at the Asn side chain. Alteration of a single nucleotide, chosen so that Asn is replaced by a different amino acid, for example, is sufficient to inactivate an N-glycosylation site. Known procedures for inactivating N-glycosylation sites in proteins include those described in U.S. Patent 5,071,972 and EP 276,846, hereby incorporated by reference.

In another example, sequences encoding Cys residues that are not essential for biological activity can be altered to cause the Cys residues to be deleted or replaced with other amino acids, preventing formation of incorrect intramolecular disulfide bridges upon renaturation. Other equivalents are prepared by modification of adjacent dibasic amino acid residues to enhance expression in yeast systems in which KEX2 protease activity is present. EP 212,914 discloses the use of site-specific mutagenesis to inactivate KEX2 protease processing sites in a protein. KEX2 protease processing sites are inactivated by deleting, adding or substituting residues to alter Arg-Arg, Arg-Lys, and Lys-Arg pairs to eliminate the occurrence of these adjacent basic residues. Lys-Lys pairings are considerably less susceptible to KEX2 cleavage, and conversion of Arg-Lys or Lys-Arg to Lys-Lys represents a conservative and preferred approach to inactivating KEX2 sites. Both murine and human flt3-L contain two KEX2 protease processing sites at amino acids 216-217 and 217-218 of SEQ ID NO:2 and at amino acids 211-212 and 212-213 of SEQ ID NO:6, respectively.

Nucleic acid sequences within the scope of the invention include isolated DNA and RNA sequences that hybridize to the native flt3-L nucleotide sequences disclosed herein under conditions of moderate or severe stringency, and which encode biologically active flt3-L. Conditions of moderate stringency, as defined by Sambrook et al. *Molecular Cloning: A Laboratory Manual,* 2 ed. Vol. 1, pp. 1.101-104, Cold Spring Harbor Laboratory Press, (1989), include use of a prewashing solution of 5 X SSC, 0.5% SDS, 1.0 mM EDTA (pH 8.0) and hybridization conditions of about 55°C, 5 X SSC, overnight. Conditions of severe stringency include higher temperatures of hybridization and washing. The skilled artisan will recognize that the temperature and wash solution salt concentration may be adjusted as necessary according to factors such as the length of the probe.

Due to the known degeneracy of the genetic code wherein more than one codon can encode the same amino acid, a DNA sequence may vary from that shown in SEQ ID NO:1 and SEQ ID NO:5 and still encode an flt3-L protein having the amino acid sequence of SEQ ID NO:2 and SEQ ID NO:6, respectively. Such variant DNA sequences may result from silent mutations (e.g., occurring during PCR amplification), or may be the product of deliberate mutagenesis of a native sequence.

The invention provides equivalent isolated DNA sequences encoding biologically active flt3-L, selected from: (a) DNA derived from the coding region of a native mammalian flt3-L gene; (b) cDNA comprising the nucleotide sequence presented in SEQ ID NO:1 or SEQ ID NO:5; (c) DNA capable of hybridization to a DNA of (a) under moderately stringent conditions and which encodes biologically active flt3-L; and (d) DNA which is degenerate as a result of the genetic code to a DNA defined in (a), (b) or (c) and which encodes biologically active flt3-L. Flt3-L proteins encoded by such DNA equivalent sequences are encompassed by the invention.

DNA that are equivalents to the DNA sequence of SEQ ID NO:1 or SEQ ID NO:5, will hybridize under moderately stringent conditions to the native DNA sequence that encode polypeptides comprising amino acid sequences of 28-163 of SEQ ID NO:2 or 28-160 of SEQ ID NO:6. Examples of flt3-L proteins encoded by such DNA, include, but are not limited to, flt3-L fragments (soluble or membrane-bound) and flt3-L proteins comprising inactivated N-glycosylation site(s), inactivated KEX2 protease processing site(s), or conservative amino acid substitution(s), as described above. Flt3-L proteins encoded by DNA derived from other mammalian species, wherein the

DNA will hybridize to the cDNA of SEQ ID NO:1 or SEQ ID NO:5, are also encompassed.

Variants possessing the requisite ability to bind flt3 receptor may be identified by any suitable assay. Biological activity of flt3-L may be determined, for example, by competition for binding to the ligand binding domain of flt3 receptor (i.e. competitive binding assays).

One type of a competitive binding assay for a flt3-L polypeptide uses a radiolabeled, soluble human flt3-L and intact cells expressing cell surface flt3 receptors. Instead of intact cells, one could substitute soluble flt3 receptors (such as a flt3:Fc fusion protein) bound to a solid phase through the interaction of a Protein A, Protein G or an antibody to the flt3 or Fc portions of the molecule, with the Fc region of the fusion protein. Another type of competitive binding assay utilizes radiolabeled soluble flt3 receptors such as a flt3:Fc fusion protein, and intact cells expressing flt3-L. Alternatively, soluble flt3-L could be bound to a solid phase to positively select flt3 expressing cells.

Competitive binding assays can be performed following conventional methodology. For example, radiolabeled flt3-L can be used to compete with a putative flt3-L homolog to assay for binding activity against surface-bound flt3 receptors. Qualitative results can be obtained by competitive autoradiographic plate binding assays, or Scatchard plots may be utilized to generate quantitative results.

Alternatively, flt3-binding proteins, such as flt3-L and anti-flt3 antibodies, can be bound to a solid phase such as a column chromatography matrix or a similar substrate suitable for identifying, separating or purifying cells that express the flt3 receptor on their surface. Binding of flt3-binding proteins to a solid phase contacting surface can be accomplished by any means, for example, by constructing a flt3-L:Fc fusion protein and binding such to the solid phase through the interaction of Protein A or Protein G. Various other means for fixing proteins to a solid phase are well known in the art and are suitable for use in the present invention. For example, magnetic microspheres can be coated with flt3-binding proteins and held in the incubation vessel through a magnetic field. Suspensions of cell mixtures containing hematopoietic progenitor or stem cells are contacted with the solid phase that has flt3-binding proteins thereon. Cells having the flt3 receptor on their surface bind to the fixed flt3-binding protein and unbound cells then are washed away. This affinity-binding method is

useful for purifying, screening or separating such flt3-expressing cells from solution. Methods of releasing positively selected cells from the solid phase are known in the art and encompass, for example, the use of enzymes. Such enzymes are preferably non-toxic and non-injurious to the cells and are preferably directed to cleaving the cell-surface binding partner. In the case of flt3:flt3-L interactions, the enzyme preferably would cleave the flt3 receptor, thereby freeing the resulting cell suspension from the "foreign" flt3-L material. The purified cell population then may be expanded *ex vivo* prior to transplantation to a patient in an amount sufficient to reconstitute the patient's hematopoietic and immune system.

Alternatively, mixtures of cells suspected of containing $flt3^+$ cells first can be incubated with a biotinylated flt3-binding protein. Incubation periods are typically at least one hour in duration to ensure sufficient binding to flt3. The resulting mixture then is passed through a column packed with avidin-coated beads, whereby the high affinity of biotin for avidin provides the binding of the cell to the beads. Use of avidin-coated beads is known in the art. See Berenson, et al. *J. Cell. Biochem.*, 10D:239 (1986). Wash of unbound material and the release of the bound cells is performed using conventional methods.

In the methods described above, suitable flt3-binding proteins are flt3-L, anti-flt3 antibodies, and other proteins that are capable of high-affinity binding of flt3. A preferred flt3-binding protein is flt3-L.

As described above, flt3-L of the invention can be used to separate cells expressing flt3 receptors. In an alternative method, flt3-L or an extracellular domain or a fragment thereof can be conjugated to a detectable moiety such as ^{125}I to detect flt3 expressing cells. Radiolabeling with ^{125}I can be performed by any of several standard methodologies that yield a functional ^{125}I-flt3-L molecule labeled to high specific activity. Or an iodinated or biotinylated antibody against the flt3 region or the Fc region of the molecule could be used. Another detectable moiety such as an enzyme that can catalyze a colorimetric or fluorometric reaction, biotin or avidin may be used. Cells to be tested for flt3 receptor expression can be contacted with labeled flt3-L. After incubation, unbound labeled flt3-L is removed and binding is measured using the detectable moiety.

The binding characteristics of flt3-L (including variants) may also be determined using the conjugated, soluble flt3 receptors (for example, ^{125}I-flt3:Fc) in competition

assays similar to those described above. In this case, however, intact cells expressing flt3 receptors, or soluble flt3 receptors bound to a solid substrate, are used to measure the extent to which a sample containing a putative flt3-L variant competes for binding with a conjugated a soluble flt3 to flt3-L.

Other means of assaying for flt3-L include the use of anti-flt3-L antibodies, cell lines that proliferate in response to flt3-L, or recombinant cell lines that express flt3 receptor and proliferate in the presenvce of flt3-L. For example, the BAF/BO3 cell line lacks the flt3 receptor and is IL-3 dependent. (See Hatakeyama, et al., *Cell*, 59: 837-845 (1989)). BAF/BO3 cells transfected with an expression vector comprising the flt3 receptor gene proliferate in response to either IL-3 or flt3-L. An example of a suitable expression vector for transfection of flt3 is the pCAV/NOT plasmid, see Mosley et al., *Cell*, 59: 335-348 (1989).

Flt3-L polypeptides may exist as oligomers, such as covalently-linked or non-covalently-linked dimers or trimers. Oligomers may be linked by disulfide bonds formed between cysteine residues on different flt3-L polypeptides. In one embodiment of the invention, a flt3-L dimer is created by fusing flt3-L to the Fc region of an antibody (e.g., IgG1) in a manner that does not interfere with binding of flt3-L to the flt3-ligand-binding domain. The Fc polypeptide preferably is fused to the C-terminus of a soluble flt3-L (comprising only the extracellular domain). General preparation of fusion proteins comprising heterologous polypeptides fused to various portions of antibody-derived polypeptides (including the Fc domain) has been described, e.g., by Ashkenazi et al. (*PNAS USA 88:*10535, 1991) and Byrn et al. (*Nature 344*:677, 1990), hereby incorporated by reference. A gene fusion encoding the flt3-L:Fc fusion protein is inserted into an appropriate expression vector. Flt3-L:Fc fusion proteins are allowed to assemble much like antibody molecules, whereupon interchain disulfide bonds form between Fc polypeptides, yielding divalent flt3-L. If fusion proteins are made with both heavy and light chains of an antibody, it is possible to form a flt3-L oligomer with as many as four flt3-L extracellular regions. Alternatively, one can link two soluble flt3-L domains with a peptide linker.

Recombinant expression vectors containing a DNA encoding flt3-L can be prepared using well known methods. The expression vectors include a flt3-L DNA sequence operably linked to suitable transcriptional or translational regulatory nucleotide sequences, such as those derived from a mammalian, microbial, viral, or insect gene. Examples of regulatory sequences include transcriptional promoters,

operators, or enhancers, an mRNA ribosomal binding site, and appropriate sequences which control transcription and translation initiation and termination. Nucleotide sequences are "operably linked" when the regulatory sequence functionally relates to the flt3-L DNA sequence. Thus, a promoter nucleotide sequence is operably linked to a flt3-L DNA sequence if the promoter nucleotide sequence controls the transcription of the flt3-L DNA sequence. The ability to replicate in the desired host cells, usually conferred by an origin of replication, and a selection gene by which transformants are identified, may additionally be incorporated into the expression vector.

In addition, sequences encoding appropriate signal peptides that are not naturally associated with flt3-L can be incorporated into expression vectors. For example, a DNA sequence for a signal peptide (secretory leader) may be fused in-frame to the flt3-L sequence so that flt3-L is initially translated as a fusion protein comprising the signal peptide. A signal peptide that is functional in the intended host cells enhances extracellular secretion of the flt3-L polypeptide. The signal peptide may be cleaved from the flt3-L polypeptide upon secretion of flt3-L from the cell.

Suitable host cells for expression of flt3-L polypeptides include prokaryotes, yeast or higher eukaryotic cells. Appropriate cloning and expression vectors for use with bacterial, fungal, yeast, and mammalian cellular hosts are described, for example, in Pouwels et al. *Cloning Vectors: A Laboratory Manual*, Elsevier, New York, (1985). Cell-free translation systems could also be employed to produce flt3-L polypeptides using RNAs derived from DNA constructs disclosed herein.

Prokaryotes include gram negative or gram positive organisms, for example, *E. coli* or *Bacilli*. Suitable prokaryotic host cells for transformation include, for example, *E. coli, Bacillus subtilis, Salmonella typhimurium*, and various other species within the genera *Pseudomonas, Streptomyces*, and *Staphylococcus*. In a prokaryotic host cell, such as *E. coli*, a flt3-L polypeptide may include an N-terminal methionine residue to facilitate expression of the recombinant polypeptide in the prokaryotic host cell. The N-terminal Met may be cleaved from the expressed recombinant flt3-L polypeptide.

Expression vectors for use in prokaryotic host cells generally comprise one or more phenotypic selectable marker genes. A phenotypic selectable marker gene is, for example, a gene encoding a protein that confers antibiotic resistance or that supplies an autotrophic requirement. Examples of useful expression vectors for prokaryotic host cells include those derived from commercially available plasmids such as the cloning

vector pBR322 (ATCC 37017). pBR322 contains genes for ampicillin and tetracycline resistance and thus provides simple means for identifying transformed cells. To construct en expression vector using pBR322, an appropriate promoter and a flt3-L DNA sequence are inserted into the pBR322 vector. Other commercially available vectors include, for example, pKK223-3 (Pharmacia Fine Chemicals, Uppsala, Sweden) and pGEM1 (Promega Biotec, Madison, WI, USA).

Promoter sequences commonly used for recombinant prokaryotic host cell expression vectors include β-lactamase (penicillinase), lactose promoter system (Chang et al., *Nature 275*:615, 1978; and Goeddel et al., *Nature 281*:544, 1979), tryptophan (trp) promoter system (Goeddel et al., *Nucl. Acids Res. 8*:4057, 1980; and EP-A-36776) and tac promoter (Maniatis, *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory, p. 412, 1982). A particularly useful prokaryotic host cell expression system employs a phage λ P_L promoter and a cI857ts thermolabile repressor sequence. Plasmid vectors available from the American Type Culture Collection which incorporate derivatives of the λ P_L promoter include plasmid pHUB2 (resident in *E. coli* strain JMB9 (ATCC 37092)) and pPLc28 (resident in *E. coli* RR1 (ATCC 53082)).

Flt3-L polypeptides alternatively may be expressed in yeast host cells, preferably from the *Saccharomyces* genus (e.g., *S. cerevisiae*). Other genera of yeast, such as *Pichia* , *K. lactis* or *Kluyveromyces*, may also be employed. Yeast vectors will often contain an origin of replication sequence from a 2μ yeast plasmid, an autonomously replicating sequence (ARS), a promoter region, sequences for polyadenylation, sequences for transcription termination, and a selectable marker gene. Suitable promoter sequences for yeast vectors include, among others, promoters for metallothionein, 3-phosphoglycerate kinase (Hitzeman et al., *J. Biol. Chem. 255*:2073, 1980) or other glycolytic enzymes (Hess et al., *J. Adv. Enzyme Reg. 7*:149, 1968; and Holland et al., *Biochem. 17*:4900, 1978), such as enolase, glyceraldehyde-3-phosphate dehydrogenase, hexokinase, pyruvate decarboxylase, phosphofructokinase, glucose-6-phosphate isomerase, 3-phosphoglycerate mutase, pyruvate kinase, triosephosphate isomerase, phosphoglucose isomerase, and glucokinase. Other suitable vectors and promoters for use in yeast expression are further described in Hitzeman, EPA-73,657 or in Fleer et. al., *Gene*, 107:285-195 (1991); and van den Berg et. al., *Bio/Technology*, 8:135-139 (1990). Another alternative is the glucose-repressible ADH2 promoter described by Russell et al. (*J. Biol. Chem.* 258:2674, 1982) and Beier et al. (*Nature* 300:724, 1982). Shuttle vectors replicable in both yeast and *E. coli* may

be constructed by inserting DNA sequences from pBR322 for selection and replication in *E. coli* (Amp^r gene and origin of replication) into the above-described yeast vectors.

The yeast α-factor leader sequence may be employed to direct secretion of the flt3-L polypeptide. The α-factor leader sequence is often inserted between the promoter sequence and the structural gene sequence. See, e.g., Kurjan et al., *Cell* 30:933, 1982; Bitter et al., *Proc. Natl. Acad. Sci. USA* 81:5330, 1984; U. S. Patent 4,546,082; and EP 324,274. Other leader sequences suitable for facilitating secretion of recombinant polypeptides from yeast hosts are known to those of skill in the art. A leader sequence may be modified near its 3' end to contain one or more restriction sites. This will facilitate fusion of the leader sequence to the structural gene.

Yeast transformation protocols are known to those of skill in the art. One such protocol is described by Hinnen et al., *Proc. Natl. Acad. Sci. USA* 75:1929, 1978. The Hinnen et al. protocol selects for Trp$^+$ transformants in a selective medium, wherein the selective medium consists of 0.67% yeast nitrogen base, 0.5% casamino acids, 2% glucose, 10 µg/ml adenine and 20 µg/ml uracil.

Yeast host cells transformed by vectors containing ADH2 promoter sequence may be grown for inducing expression in a "rich" medium. An example of a rich medium is one consisting of 1% yeast extract, 2% peptone, and 1% glucose supplemented with 80 µg/ml adenine and 80 µg/ml uracil. Derepression of the ADH2 promoter occurs when glucose is exhausted from the medium.

Mammalian or insect host cell culture systems could also be employed to express recombinant flt3-L polypeptides. Baculovirus systems for production of heterologous proteins in insect cells are reviewed by Luckow and Summers, *Bio/Technology* 6:47 (1988). Established cell lines of mammalian origin also may be employed. Examples of suitable mammalian host cell lines include the COS-7 line of monkey kidney cells (ATCC CRL 1651) (Gluzman et al., *Cell* 23:175, 1981), L cells, C127 cells, 3T3 cells (ATCC CCL 163), Chinese hamster ovary (CHO) cells, HeLa cells, and BHK (ATCC CRL 10) cell lines, and the CV-1/EBNA-1 cell line derived from the African green monkey kidney cell line CVI (ATCC CCL 70) as described by McMahan et al. (*EMBO J.* 10: 2821, 1991).

Transcriptional and translational control sequences for mammalian host cell expression vectors may be excised from viral genomes. Commonly used promoter

sequences and enhancer sequences are derived from Polyoma virus, Adenovirus 2, Simian Virus 40 (SV40), and human cytomegalovirus. DNA sequences derived from the SV40 viral genome, for example, SV40 origin, early and late promoter, enhancer, splice, and polyadenylation sites may be used to provide other genetic elements for expression of a structural gene sequence in a mammalian host cell. Viral early and late promoters are particularly useful because both are easily obtained from a viral genome as a fragment which may also contain a viral origin of replication (Fiers et al., *Nature* 273:113, 1978). Smaller or larger SV40 fragments may also be used, provided the approximately 250 bp sequence extending from the *Hind* III site toward the *Bgl* I site located in the SV40 viral origin of replication site is included.

Exemplary expression vectors for use in mammalian host cells can be constructed as disclosed by Okayama and Berg (*Mol. Cell. Biol.* 3:280, 1983). A useful system for stable high level expression of mammalian cDNAs in C127 murine mammary epithelial cells can be constructed substantially as described by Cosman et al. (*Mol. Immunol.* 23:935, 1986). A useful high expression vector, PMLSV N1/N4, described by Cosman et al., *Nature* 312:768, 1984 has been deposited as ATCC 39890. Additional useful mammalian expression vectors are described in EP-A-0367566, and in U.S. Patent Application Serial No. 07/701,415, filed May 16, 1991, incorporated by reference herein. The vectors may be derived from retroviruses. In place of the native signal sequence, a heterologous signal sequence may be added, such as the signal sequence for IL-7 described in United States Patent 4,965,195; the signal sequence for IL-2 receptor described in Cosman et al., *Nature* 312:768 (1984); the IL-4 signal peptide described in EP 367,566; the type I IL-1 receptor signal peptide described in U.S. Patent 4,968,607; and the type II IL-1 receptor signal peptide described in EP 460,846.

Flt3-L as an isolated or homogeneous protein according to the invention may be produced by recombinant expression systems as described above or purified from naturally occurring cells. Flt3-L can be purified to substantial homogeneity, as indicated by a single protein band upon analysis by SDS-polyacrylamide gel electrophoresis (SDS-PAGE).

One process for producing flt3-L comprises culturing a host cell transformed with an expression vector comprising a DNA sequence that encodes flt3-L under conditions sufficient to promote expression of flt3-L. Flt3-L is then recovered from culture medium or cell extracts, depending upon the expression system employed. As

is known to the skilled artisan, procedures for purifying a recombinant protein will vary according to such factors as the type of host cells employed and whether or not the recombinant protein is secreted into the culture medium.

For example, when expression systems that secrete the recombinant protein are employed, the culture medium first may be concentrated using a commercially available protein concentration filter, for example, an Amicon or Millipore Pellicon ultrafiltration unit. Following the concentration step, the concentrate can be applied to a purification matrix such as a gel filtration medium. Alternatively, an anion exchange resin can be employed, for example, a matrix or substrate having pendant diethylaminoethyl (DEAE) groups. The matrices can be acrylamide, agarose, dextran, cellulose or other types commonly employed in protein purification. Alternatively, a cation exchange step can be employed. Suitable cation exchangers include various insoluble matrices comprising sulfopropyl or carboxymethyl groups. Sulfopropyl groups are preferred. Finally, one or more reversed-phase high performance liquid chromatography (RP-HPLC) steps employing hydrophobic RP-HPLC media, (e.g., silica gel having pendant methyl or other aliphatic groups) can be employed to further purify flt3-L. Some or all of the foregoing purification steps, in various combinations, are well known and can be employed to provide a substantially homogeneous recombinant protein.

It is possible to utilize an affinity column comprising the ligand binding domain of flt3 receptors to affinity-purify expressed flt3-L polypeptides. Flt3-L polypeptides can be removed from an affinity column using conventional techniques, e.g., in a high salt elution buffer and then dialyzed into a lower salt buffer for use or by changing pH or other components depending on the affinity matrix utilized. Alternatively, the affinity column may comprise an antibody that binds flt3-L. Example 6 describes a procedure for employing flt3-L of the invention to generate monoclonal antibodies directed against flt3-L.

Recombinant protein produced in bacterial culture is usually isolated by initial disruption of the host cells, centrifugation, extraction from cell pellets if an insoluble polypeptide, or from the supernatant fluid if a soluble polypeptide, followed by one or more concentration, salting-out, ion exchange, affinity purification or size exclusion chromatography steps. Finally, RP-HPLC can be employed for final purification steps. Microbial cells can be disrupted by any convenient method, including freeze-thaw cycling, sonication, mechanical disruption, or use of cell lysing agents.

Transformed yeast host cells are preferably employed to express flt3-L as a secreted polypeptide in order to simplify purification. Secreted recombinant polypeptide from a yeast host cell fermentation can be purified by methods analogous to those disclosed by Urdal et al. (*J. Chromatog.* 296:171, 1984). Urdal et al. describe two sequential, reversed-phase HPLC steps for purification of recombinant human IL-2 on a preparative HPLC column.

Antisense or sense oligonucleotides comprising a single-stranded nucleic acid sequence (either RNA or DNA) capable of binding to a target flt3-L mRNA sequence (forming a duplex) or to the flt3-L sequence in the double-stranded DNA helix (forming a triple helix) can be made according to the invention. Antisense or sense oligonucleotides, according to the present invention, comprise a fragment of the coding region of flt3-L cDNA. Such a fragment generally comprises at least about 14 nucleotides, preferably from about 14 to about 30 nucleotides. The ability to create an antisense or a sense oligonucleotide, based upon a cDNA sequence for a given protein is described in, for example, Stein and Cohen, *Cancer Res.* 48:2659, 1988 and van der Krol et al., *BioTechniques* 6:958, 1988.

Binding of antisense or sense oligonucleotides to target nucleic acid sequences results in the formation of complexes that block translation (RNA) or transcription (DNA) by one of several means, including enhanced degradation of the duplexes, premature termination of transcription or translation, or by other means. The antisense oligonucleotides thus may be used to block expression of flt3-L proteins. Antisense or sense oligonucleotides further comprise oligonucleotides having modified sugar-phosphodiester backbones (or other sugar linkages, such as those described in WO91/06629) and wherein such sugar linkages are resistant to endogenous nucleases. Such oligonucleotides with resistant sugar linkages are stable *in vivo* (i.e., capable of resisting enzymatic degradation) but retain sequence specificity to be able to bind to target nucleotide sequences. Other examples of sense or antisense oligonucleotides include those oligonucleotides which are covalently linked to organic moieties, such as those described in WO 90/10448, and other moieties that increases affinity of the oligonucleotide for a target nucleic acid sequence, such as poly-(L-lysine). Further still, intercalating agents, such as ellipticine, and alkylating agents or metal complexes may be attached to sense or antisense oligonucleotides to modify binding specificities of the antisense or sense oliginucleotide for the target nucleotide sequence.

Antisense or sense oligonucleotides may be introduced into a cell containing the target nucleic acid sequence by any gene transfer method, including, for example, $CaPO_4$-mediated DNA transfection, electroporation, or by using gene transfer vectors such as Epstein-Barr virus. Antisense or sense oligonucleotides are preferably introduced into a cell containing the target nucleic acid sequence by insertion of the antisense or sense oligonucleotide into a suitable retroviral vector, then contacting the cell with the retrovirus vector containing the inserted sequence, either *in vivo* or *ex vivo*. Suitable retroviral vectors include, but are not limited to, the murine retrovirus M-MuLV, N2 (a retrovirus derived from M-MuLV), or or the double copy vectors designated DCT5A, DCT5B and DCT5C (see PCT Application US 90/02656).

Sense or antisense oligonucleotides also may be introduced into a cell containing the target nucleotide sequence by formation of a conjugate with a ligand binding molecule, as described in WO 91/04753. Suitable ligand binding molecules include, but are not limited to, cell surface receptors, growth factors, other cytokines, or other ligands that bind to cell surface receptors. Preferably, conjugation of the ligand binding molecule does not substantially interfere with the ability of the ligand binding molecule to bind to its corresponding molecule or receptor, or block entry of the sense or antisense oligonucleotide or its conjugated version into the cell.

Alternatively, a sense or an antisense oligonucleotide may be introduced into a cell containing the target nucleic acid sequence by formation of an oligonucleotide-lipid complex, as described in WO 90/10448. The sense or antisense oligonucleotide-lipid complex is preferably dissociated within the cell by an endogenous lipase.

Flt3-L polypeptides of the invention can be formulated according to known methods used to prepare pharmaceutically useful compositions. Flt3-L can be combined in admixture, either as the sole active material or with other known active materials, with pharmaceutically suitable diluents (e.g., Tris-HCl, acetate, phosphate), preservatives (e.g., Thimerosal, benzyl alcohol, parabens), emulsifiers, solubilizers, adjuvants and/or carriers. Suitable carriers and their formulations are described in Remington's Pharmaceutical Sciences, 16th ed. 1980, Mack Publishing Co. In addition, such compositions can contain flt3-L complexed with polyethylene glycol (PEG), metal ions, or incorporated into polymeric compounds such as polyacetic acid, polyglycolic acid, hydrogels, etc., or incorporated into liposomes, microemulsions, micelles, unilamellar or multilamellar vesicles, erythrocyte ghosts or spheroblasts. Such compositions will influence the physical state, solubility, stability, rate of *in vivo*

release, and rate of *in vivo* clearance of flt3-L. Flt3-L can also be conjugated to antibodies against tissue-specific receptors, ligands or antigens, or coupled to ligands of tissue-specific receptors. Where the flt3 receptor is found on neoplastic cells, the flt3-L may be conjugated to a toxin whereby flt3-L is used to deliver the toxin to the specific site, or may be used to sensitize such neoplastic cells to subsequently administered anti-neoplastic agents.

Flt3-L can be administered topically, parenterally, or by inhalation. The term "parenteral" includes subcutaneous injections, intravenous, intramuscular, intracisternal injection, or infusion techniques. These compositions will typically contain an effective amount of the flt3-L, alone or in combination with an effective amount of any other active material. Such dosages and desired drug concentrations contained in the compositions may vary depending upon many factors, including the intended use, patient's body weight and age, and route of administration. Preliminary doses can be determined according to animal tests, and the scaling of dosages for human administration can be performed according to art-accepted practices. Keeping the above description in mind, typical dosages of flt3-L may range from about 10 µg per square meter to about 1000 µg per square meter. A preferred dose range is on the order of about 100 µg per square meter to about 300 µg per square meter.

In addition to the above, the following examples are provided to illustrate particular embodiments and not to limit the scope of the invention.

EXAMPLE 1

Preparation of Flt3-Receptor:Fc Fusion Protein

This example describes the cloning of murine flt3 cDNA, and the construction of an expression vector encoding a soluble murine flt3-receptor:Fc fusion protein for use in detecting cDNA clones encoding flt3-L. Polymerase chain reaction (PCR) cloning of the flt3 cDNA from a murine T-cell was accomplished using the oligonucleotide primers and the methods as described by Lyman et al., *Oncogene*, 8:815-822, (1993), incorporated herein by reference. The cDNA sequence and encoded amino acid sequence for mouse flt3 receptor is presented by Rosnet et el., *Oncogene*, 6:1641-1650, (1991), hereby incorporated by reference. The mouse flt3 protein has a 542 amino acid extracellular domain, a 21 amino acid transmembrane domain, and a 437 amino acid cytoplasmic domain.

Prior to fusing the murine flt3 cDNA to the N-terminus of cDNA encoding the Fc portion of a human IgG1 molecule, the amplified mouse flt3 cDNA fragment was inserted into Asp718-*Not*I site of pCAV/NOT, described in PCT Application WO 90/05183. DNA encoding a single chain polypeptide comprising the Fc region of a human IgG1 antibody was cloned into the *Spe*I site of the pBLUESCRIPT SK® vector, which is commercially available from Stratagene Cloning Systems, La Jolla, California. This plasmid vector is replicable in *E. coli* and contains a polylinker segment that includes 21 unique restriction sites. A unique *Bgl*II site was introduced near the 5' end of the inserted Fc encoding sequence, such that the *Bgl*II site encompasses the codons for amino acids three and four of the Fc polypeptide.

The encoded Fc polypeptide extends from the N-terminal hinge region to the native C-terminus, i.e., is an essentially full-length antibody Fc region. Fragments of Fc regions, e.g., those that are truncated at the C-terminal end, also may be employed. The fragments preferably contain multiple cysteine residues (at least the cysteine residues in the hinge reaction) to permit interchain disulfide bonds to form between the Fc polypeptide portions of two separate flt3:Fc fusion proteins, forming dimers as discussed above.

An Asp718 restriction endonuclease cleavage site was introduced upstream of the flt3 coding region. An Asp 718-*Not*I fragment of mouse flt3 cDNA (comprising the entire extracellular domain, the transmembrane region, and a small portion of the cytoplasmic domain) was isolated. The above-described Asp718-*Not*I flt3 partial cDNA was cloned into the pBLUESCRIPT SK® vector containing the Fc cDNA, such that the flt3 cDNA is positioned upstream of the Fc cDNA. Single stranded DNA derived from the resulting gene fusion was mutagenized by the method described in Kunkel (*Proc. Natl. Acad. Sci. USA* 82:488, 1985) and Kunkel et al. (*Methods in Enzymol.* 154:367, 1987) in order to perfectly fuse the entire extracellular domain of flt3 to the Fc sequence. The mutagenized DNA was sequenced to confirm that the proper nucleotides had been removed (i.e., transmembrane region and partial cytoplasmic domain DNA was deleted) and that the flt3 and Fc sequences were in the same reading frame. The fusion cDNA was then excised and inserted into a mammalian expression vector designated sfHAV-EO 409 which was cut with *Sal*I-*Not*I, and the *Sal*I and Asp718 ends blunted. The sfHAV-EO vector (also known as pDC406) is described by McMahan et al. (*EMBO J.*, 10; No. 10: 2821-2832 (1991)).

Flt3:Fc fusion proteins preferably are synthesized in recombinant mammalian cell culture. The flt3:Fc fusion-containing expression vector was transfected into CV-1 cells (ATCC CCL 70) and COS-7 cells (ATCC CRL 1651), both derived from monkey kidney. Flt3:Fc expression level was relatively low in both CV-1 and COS-7 cells. Thus, expression in 293 cells (transformed primary human embryonal kidney cells, ATCC CRL 1573) was attempted.

The 293 cells transfected with the sfHAV-EO/flt3:Fc vector were cultivated in roller bottles to allow transient expression of the fusion protein, which is secreted into the culture medium *via* the flt3 signal peptide. The fusion protein was purified on protein A Sepharose columns, eluted, and used to screen cells for the ability to bind flt3:Fc, as described in Examples 2 and 3.

EXAMPLE 2
Screening Cells for Flt3:Fc Binding

Approximately 100 different primary cells and cell lines falling into the following general categories: primary murine fetal brain cells, murine fetal liver cell lines, rat fetal brain cell lines, human lung carcinoma (fibroblastoid) cell lines, human and murine lymphoid and myeloid cell lines were assayed for flt3:Fc binding. Cell lines were incubated with flt3:Fc, followed by a biotinylated anti-human Fc antibody, followed by streptavidin-phycoerythrin (Becton Dickinson). The biotinylated antibody was purchased from Jackson Immunoresearch Laboratories. Streptavidin binds to the biotin molecule attached to the anti-human Fc antibody, which in turn binds to the Fc portion of the flt3:Fc fusion protein. Phycoerythrin is a fluorescent phycobiliprotein which serves as a detectable label. The level of fluorescence signal was measured for each cell type using a FACScan® flow cytometer (Becton Dickinson). The cell types deemed positive for flt3:Fc binding were identified.

EXAMPLE 3
Isolation and Cloning of Flt3 L cDNA from Murine T-Cell cDNA Library

A murine T-cell cDNA library of cell line P7B-0.3A4 was chosen as a possible source of flt3-L cDNA. P7B-0.3A4 is a murine T cell clone that is Thy1.2^{+}, CD4^{-}, CD8^{-}, TCR*ab*$^{\pm}$, CD44^{+}. It was originally cloned at a cell density of 0.33 cells/well in the presence of rHuIL-7 and immobilized anti-CD3 MAb, and was grown in

continuous culture for more than 1 year by passage once a week in medium containing 15 ng/ml rHuIL-7. The parent cell line was derived from lymph node cells of SJL/J mice immunized with 50 nmoles $PLP_{139-151}$ peptide and 100 µg *Mycobacterium tuberculosis* H37Ra in Incomplete Freund's Adjuvant. PLP is the proteolipid protein component of the myelin sheath of the central nervous system. The peptide composed of amino acids 139-151 has previously been shown to be the encephalogenic peptide in experimental autoimmune encephalomyelitis (EAE), a murine model for multiple sclerosis in SJL/J mice. (Touhy, V.K., Z. Lu, R.A. Sobel, R.A. Laursen and M.B. Lees; 1989. Identification of an encephalitogenic determinant of myelin proteolipid protein for SJL mice. *J. Immunol.* 142:1523.) After the initial culture in the presence of antigen, the parent cell line, designated PLP7, had been in continuous culture with rHuIL-7 (and without antigen) for more than 6 months prior to cloning.

P7B-0.3A4 proliferates only in response to very high concentrations of $PLP_{139-151}$ peptide in the presence of irradiated syngeneic splenocytes and is not encephalogenic or alloresponsive. This clone proliferates in response to immobilized anti-CD3 MAb, IL-2, and IL-7, but not IL-4.

Binding of flt3:Fc was observed on murine T-cells and human T-cells, and therefore a murine T-cell line was chosen (0.3A4) due to its ease of growth. A murine 0.3A4 cDNA library in sfHAV-EO was prepared as described in McMahan et al. (*EMBO J.*, 10; No:10; 2821-2832 1991). sfHAV-EO is a mammalian expression vector that also replicates in *E. coli*. sfHAV-EO contains origins of replication derived from SV40, Epstein-Barr virus and pBR322 and is a derivative of HAV-EO described by Dower et al., *J.Immunol.* 142:4314 (1989). sfHAV-EO differs from HAV-EO by the deletion of the intron present in the adenovirus 2 tripartite leader sequence in HAV-EO. Briefly, murine T-cell cDNA was cloned into the *Sal*I site of sfHAV-EO by an adaptor method similar to that described by Haymerle et al (*Nucl. Acids Res.* 14:8615, 1986), using the following oligonucleotide adapter pair:

5' TCGACTGGAACGAGACGACCTGCT 3' SEQ ID NO:3
3' GACCTTGCTCTGCTGGACGA 5' SEQ ID NO:4

Double-stranded, blunt-ended, random-primed cDNA was prepared from 0.3A4 poly (A)+ RNA essentially as described by Gubler and Hoffman, *Gene*, 25:263-269 (1983), using a Pharmacia DNA kit. The above adapters were added to the cDNA as described by Haymerle et al.. Low molecular weight material was removed by passage over

Sephacryl S-1000 at 65 °C, and the cDNA was ligated into sfHAV-EO410, which had previously been cut with *SalI* and ligated to the same oligonucleotide pair. This vector is designated as sfHAV-EO410. DNA was electroporated (Dower et al., *Nucleic Acids Res.*, 16:6127-6145, (1988) into *E. coli* DH10B, and after one hour growth at 37 °C, the transformed cells were frozen in one milliliter aliquots in SOC medium (Hanahan et al., *J. Mol. Biol.*, 166:557-580, (1983) containing 20% glycerol. One aliquot was titered to determine the number of ampcillin-resistant colonies. The resulting 0.3A4 library had 1.84 million clones.

E. coli strain DH10B cells transfected with the cDNA library in sfHAV-EO410 were plated to provide approximately 1600 colonies per plate. Colonies were scraped from each plate, pooled, and plasmid DNA prepared from each pool. The pooled DNA, representing about 1600 colonies, was then used to transfect a sub-confluent layer of CV-1/EBNA-1 cells using DEAE-dextran followed by chloroquine treatment, similar to that described by Luthman et al., *Nucl. Acids Res.* 11:1295, (1983) and McCutchan et al., *J. Natl. Cancer Inst.* 41:351, (1986). The CV-1/EBNA-1 cell line (ATCC CRL10478) constitutively expresses EBV nuclear antigen-1 driven from the CMV immediate-early enhancer/promoter. CV1-EBNA-1 was derived from the African Green Monkey kidney cell line CV-1 (ATCC CCL 70), as described by McMahan et al. (*EMBO J.* 10:2821, 1991).

In order to transfect the CV-1/EBNA-1 cells with the cDNA library, the cells were maintained in complete medium (Dulbecco's modified Eagle's media (DMEM) containing 10% (v/v) fetal calf serum (FCS), 50 U/ml penicillin, 50 U/ml streptomycin, 2 mM L-glutamine) and were plated at a density of about 2 x 10^5 cells/well on single-well chambered slides (Lab-Tek). Slides were pretreated with 1 ml human fibronectin (10 µg/ml in PBS) for 30 minutes followed by 1 wash with PBS. Media was removed from the adherent cell layer and replaced with 1.5 ml complete medium containing 66.6 µM chloroquine sulfate. Two-tenths ml of DNA solution (2 µg DNA, 0.5 mg/ml DEAE-dextran in complete medium containing chloroquine) was then added to the cells and incubated for 5 hours. Following the incubation, the media was removed and the cells shocked by addition of complete medium containing 10% DMSO for 2.5 to 20 minutes followed by replacement of the solution with fresh complete medium. The cells were cultured for 2 to 3 days to permit transient expression of the inserted sequences.

Transfected monolayers of CV-1/EBNA-1 cells were assayed for expression of flt3-L by slide autoradiography essentially as described by Gearing et al. (*EMBO J.* 8:3667, 1989). Transfected CV-1/EBNA-1 cells (adhered to chambered slides) were washed once with binding medium with nonfat dry milk (BM-NFDM) (RPMI medium 1640 containing 25 mg/ml bovine serum albumin (BSA), 2 mg/ml sodium azide, 20 mM HEPES, pH 7.2, and 50 mg/ml nonfat dry milk). Cells were then incubated with flt3:Fc in BM-NFDM (1 μg/ml) for 1 hour at room temperature. After incubation, the cell monolayers in the chambered slides were washed three times with BM-NFDM to remove unbound flt3:Fc fusion protein and then incubated with 40 ng/ml ^{125}I-mouse anti-human Fc antibody (described below) (a 1:50 dilution) for 1 hour at room temperature. The cells were washed three times with BM-NFDM, followed by 2 washes with phosphate-buffered saline (PBS) to remove unbound ^{125}I-mouse anti-human Fc antibody. The cells were fixed by incubating for 30 minutes at room temperature in 2.5% glutaraldehyde in PBS, pH 7.3, washed twice in PBS and air dried. The chamber slides containing the cells were exposed on a Phophorimager (Molecular Dynamics) overnight, then dipped in Kodak GTNB-2 photographic emulsion (6x dilution in water) and exposed in the dark for 3-5 days at 4 °C in a light proof box. The slides were then developed for approximately 4 minutes in Kodak D19 developer (40 g/500 ml water), rinsed in water and fixed in Agfa G433C fixer. The slides were individually examined with a microscope at 25-40x magnification and positive cells expressing flt3-L were identified by the presence of autoradiographic silver grains against a light background.

The mouse anti-human Fc antibody was obtained from Jackson Laboratories. This antibody showed minimal binding to Fc proteins bound to the Fcγ receptor. The antibody was labeled using the Chloramine T method. Briefly, a Sephadex G-25 column was prepared according to the manufacturer's instructions. The column was pretreated with 10 column volumes of PBS containing 1% bovine serum albumin to reduce nonspecific adsorption of antibody to the column and resin. Nonbound bovine serum albumin was then washed from the column with 5 volumes of PBS lacking bovine serum albumin. In a microfuge tube 10 μg of antibody (dissolved in 10 μl of PBS) was added to 50 μl of 50 mM sodium phosphate buffer (pH 7.2) 2.0 mCi of carrier-free Na^{125}I was added and the solution was mixed well. 15 μl of a freshly prepared solution of chloramine-T (2 mg/ml in 0.1 M sodium phosphate buffer (pH 7.2)) was then added and the mixture was incubated for 30 minutes at room temperature, and the mixture then was immediately applied to the column of Sephadex G-25. The radiolabelled antibody was then eluted from the column by collecting 100-

150 µl fractions of eluate. Bovine serum albumin was added to the eluted fractions containing the radiolabeled antibody to a final concentration of 1%. Radioiodination yielded specific activities in the range of 5-10 x 10^{15} cpm/nmol protein.

Using the slide autoradiography approach, the approximately 1,840,000 cDNAs were screened in pools of approximately 1,600 cDNAs until assay of one transfectant pool showed multiple cells clearly positive for flt3:Fc binding. This pool was then partitioned into pools of 500 and again screened by slide autoradiography and a positive pool was identified. This pool was partitioned into pools of 100 and again screened. Individual colonies from this pool of 100 were screened until a clone (clone #6C) was identified which directed synthesis of a surface protein with detectable flt3:Fc binding activity. This clone was isolated, and its 0.88kb cDNA insert was sequenced.

The nucleotide and encoded amino acid sequences of the coding region of the murine flt3-ligand cDNA of clone #6C are presented in SEQ ID NOs:1 and 2. The cDNA insert is 0.88kb in length. The open-reading frame within this sequence could encode a protein of 231 amino acids. Thus, DNA and encoded amino acid sequences for the 231-amino acid open reading frame are presented in SEQ ID NOs:1 and 2. The protein of SEQ ID NO:2 is a type I transmembrane protein, with an N-terminal signal peptide (amino acids 1 to 27), an extracellular domain (amino acids 28-188) a transmembrane domain (amino acids 189-211) and a cytoplasmic domain (amino acids 212-231). The predicted molecular weight of the native protein following cleavage of the signal sequence is 23,164 daltons. The mature protein has an estimated pI of 9.372. There are 56 bp of 5' noncoding sequence and 126 bp of 3' non-coding sequence flanking the coding region, including the added cDNA adapters. The above-described cloning procedure also produced a murine flt3 ligand clone #5H, which is identical to the #6C clone beginning at nucleotide 49 and continuing through nucleotide 545 (corresponding to amino acid 163) of SEQ ID NO:1. The #5H clone completely differs from that point onward, and represents an alternate splicing construct.

The vector sfHAV-EO410 containing the flt3-L cDNA in *E. coli* DH10B cells was deposited with the American Type Culture Collection, Rockville, MD, USA (ATCC) on April 20, 1993 and assigned accession number ATCC 69286. The deposit was made under the terms of the Budapest Treaty.

EXAMPLE 4
Cloning of cDNA Encoding Human Flt3-L

A cDNA encoding human flt3-L was cloned from a human clone 22 T cell λgt10 random primed cDNA library as described by Sims et al., *PNAS*, 86:8946-8950 (1989). The library was screened with a 413 bp Ple I fragment corresponding to the extracellular domain of the murine flt3-L (nucleotides 103-516 of SEQ ID NO:1). The fragment was random primed, hybridized overnight to the library filters at 55°C in oligo prehybridization buffer. The fragment was then washed at 55°C at 2 x SSC/0.1% SDS for one hour, followed by 1 x SSC/0.1% SDS for one hour and then by 0.5 x SSC/0,1% SDS for one hour. The DNA from the positive phage plaques was extracted, and the inserts were amplified by PCR using oligonucleotides specific for the phage arms. The DNA then was sequenced, and the sequence for clone #9 is shown in SEQ ID NO:5. Additional human flt3-L cDNA was isolated from the same λgt10 random primed cDNA library as described above by screening the library with a fragment of the extracellular domain of the murine clone #5H cDNA comprising a cDNA sequence essentially corresponding to nucleotides 128-541 of SEQ ID NO:1.

Sequencing of the 988 bp cDNA clone #9 revealed an open reading frame of 705 bp surrounded by 29 bp of 5' non-coding sequence and 250 bp of 3' non-coding sequence. The 3' non-coding region did not contain a poly-A tail. There were no in-frame stop codons upstream of the initiator methionine. The open reading frame encodes a type I transmembrane protein of 235 amino acids as shown by amino acids 1-235 of SEQ ID NO:6. The protein has an N-terminal signal peptide of alternatively 26 or 27 amino acids. There exists a slightly greater probability that the N-terminal signal peptide is 26 amino acids in length than 27 amino acids in length. The signal peptide is followed by a 156 or a 155 amino acid extracellular domain (for signal peptides of 26 and 27 amino acids, respectively); a 23 amino acid transmembrane domain and a 30 amino acid cytoplasmic domain. Human flt3-L shares overall 72% amino acid identity and 78% amino acid similarity with murine flt3-L. The vector pBLUESCRIPT SK(-) containing the human flt3-L cDNA of clone #9 was deposited with the American Type Culture Collection, Rockville, Maryland, USA (ATCC) on August 6, 1993 and assigned acession number ATCC 69382. The deposit was made under the terms of the Budapest Treaty.

EXAMPLE 5
Expression of Flt3-L in Yeast

For expression of soluble flt3-L in yeast, synthetic oligonucleotide primers were used to amplify via PCR (Mullis and Faloona, *Meth. Enzymol.* 155:335-350, 1987) the entire extracellular coding domain of flt3-L between the end of the signal peptide and the start of the transmembrane segment. The 5' primer (5'-AATTGGTACCTTTGGATAAAAGAGACTACAAGGACGACGATGACAAGACA-CCTGACTGTTACTTCAGCCAC-3') SEQ ID NO:7 encoded a portion of of the alpha factor leader and an antigenic octapeptide, the FLAG sequence fused in-frame with the predicted mature N-terminus of flt3-L. The 3' oligonucleotide (5'-ATATGGATC-CCTACTGCCTGGGCCGAGGCTCTGGGAG-3') SEQ ID NO:8 created a termination codon following Gln-189, just at the putative transmembrane region. The PCR-generated DNA fragment was ligated into a yeast expression vector (for expression in *K. lactis*) that directs secretion of the recombinant product into the yeast medium (Fleer et. al., *Gene*, 107:285-195 (1991); and van den Berg et. al., *Bio/Technology*, 8:135-139 (1990)). The FLAG:flt3-L fusion protein was purified from yeast broth by affinity chromotography as previously described (Hopp et. al., *Biotechnology*, 6:1204-1210, 1988).

EXAMPLE 6
Monoclonal Antibodies to Flt3-L

This example illustrates a method for preparing monoclonal antibodies to flt3-L. Flt3-L is expressed in mammalian host cells such as COS-7 or CV-1/EBNA-1 cells and purified using flt3:Fc affinity chromatography. Purified flt3-L, a fragment thereof such as the extracellular domain, synthetic peptides or cells that express flt3-L can be used to generate monoclonal antibodies against flt3-L using conventional techniques, for example, those techniques described in U.S. Patent 4,411,993. Briefly, mice are immunized with flt3-L as an immunogen emulsified in complete Freund's adjuvant, and injected in amounts ranging from 10-100 µg subcutaneously or intraperitoneally. Ten to twelve days later, the immunized animals are boosted with additional flt3-L emulsified in incomplete Freund's adjuvant. Mice are periodically boosted thereafter on a weekly to bi-weekly immunization schedule. Serum samples are periodically taken by retro-orbital bleeding or tail-tip excision to test for flt3-L antibodies by dot blot assay, ELISA (Enzyme-Linked Immunosorbent Assay) or inhibition of flt3 binding.

Following detection of an appropriate antibody titer, positive animals are provided one last intravenous injection of flt3-L in saline. Three to four days later, the animals are sacrificed, spleen cells harvested, and spleen cells are fused to a murine myeloma cell line, e.g., NS1 or preferably P3x63Ag8.653 (ATCC CRL 1580). Fusions generate hybridoma cells, which are plated in multiple microtiter plates in a HAT (hypoxanthine, aminopterin and thymidine) selective medium to inhibit proliferation of non-fused cells, myeloma hybrids, and spleen cell hybrids.

The hybridoma cells are screened by ELISA for reactivity against purified flt3-L by adaptations of the techniques disclosed in Engvall et al., *Immunochem.* 8:871, 1971 and in U.S. Patent 4,703,004. A preferred screening technique is the antibody capture technique described in Beckmann et al., (*J. Immunol.* 144:4212, 1990) Positive hybridoma cells can be injected intraperitoneally into syngeneic BALB/c mice to produce ascites containing high concentrations of anti-flt3-L-L monoclonal antibodies. Alternatively, hybridoma cells can be grown *in vitro* in flasks or roller bottles by various techniques. Monoclonal antibodies produced in mouse ascites can be purified by ammonium sulfate precipitation, followed by gel exclusion chromatography. Alternatively, affinity chromatography based upon binding of antibody to protein A or protein G can also be used, as can affinity chromatography based upon binding to flt3 L.

EXAMPLE 7

Use of Flt3-L Alone and in Combination with IL-7 or IL-3

This example demonstrates the stimulation and proliferation of $AA4.1^+$ fetal liver cells by compositions containing flt3-L and IL-7; as well as the stimulation and proliferation of c-*kit*-positive (c-kit^+) cells by compositions containing flt3-L and IL-3.

AA4.1-positive ($AA4.1^+$) expressing cells were isolated from the livers of day 14 fetal C57BL/6 mice by cell panning in Optilux 100 mm plastic Petri dishes (Falcon No. 1001, Oxnard, CA). Plates were coated overnight at 4 °C in PBS plus 0.1% fetal bovine serum (FBS) containing 10 µg/ml AA4.1 antibody (McKearn et. al., *J. Immunol.*, 132:332-339, 1984) and then washed extensively with PBS plus 1% FBS prior to use. A single cell suspension of liver cells was added at 10^7 cells/dish in PBS plus 1% FBS and allowed to adhere to the plates for two hours at 4 °C. The plates were then extensively washed, and the adhering cells were harvested by scraping for

analysis or further use in the hematopoiesis assays described below. FACS analysis using AA4.1 antibody demonstrated a >95% AA4.1$^+$ cell population.

C-*kit*$^+$ pluripotent stem cells were purified from adult mouse bone marrow (de Vries et. al. , *J. Exp. Med.*, 176:1503-1509, 1992; and Visser and de Vries, *Methods in Cell Biol.*, 1993, submitted). Low density cells (≤ 1.078 g/cm^3) positive for the lectin wheat germ agglutinin and negative for the antigens recognized by the B220 and 15-1.4.1 (Visser et. al., *Meth. in Cell Biol.*, 33:451-468, 1990) monoclonal antibodies, could be divided into sub-populations of cells that do and do not express c-*kit* by using biotinylated Steel factor. The c-*kit*$^+$fraction has been shown to contain pluripotent hematopoietic stem cells (de Vries et. al., *Science* 255:989-991, 1992; Visser and de Vries, *Methods in Cell Biol.*, 1993, submitted; and Ware et. al., 1993, submitted).

AA4.1+ Fetal liver cells were cultured in recombinant IL-7 (U.S. Patent No. 4,965,195) at 100 ng/ml and recombinant flt3-L at 250 ng/ml. Flt3-L was used in three different forms in the experiments: (1) as present on fixed, flt3-L-transfected CV1/EBNA cells; (2) as concentrated culture supernatants from these same flt3-L-transfected CV1/EBNA cells; and (3) as a purified and isolated polypeptide preparation from yeast supernatant as described in Example 5.

Hematopoiesis Assays

The proliferation of c-*kit* $^+$ stem cells, fetal liver AA4.1$^+$ cells was assayed in [3H]-thymidine incorporation assays as essentially described by deVries et. al., *J. Exp. Med.*, 173:1205-1211, 1991. Purified c-*kit*$^+$ stem cells were cultured at 37 °C in a fully humidified atmosphere of 6.5% CO_2 and 7% O_2 in air for 96 hours. Murine recombinant IL-3 was used at a final concentration of 100 ng/ml. Subsequently, the cells were pulsed with 2 μCi per well of [^{3}H]-thymidine (81 Ci/mmol; Amersham Corp., Arlington Heights, IL) and incubated for an additional 24 hours. AA4.1$^+$ cells (approximately 20,000 cells/well) were incubated in IL-7, flt3-L and flt3-L + IL-7 for 48 hours, followed by [^{3}H]-thymidine pulse of six hours. The results of flt3-L and IL-7 are shown in Table I, and results of flt3-L and IL-3 are shown in Table II.

TABLE I

Effect of Flt3-L and IL-7 on Proliferation of AA4.1+ Fetal Liver Cells.

	Factor			
	Control	flt3-L	IL-7	flt3-L + IL-7
[^{3}H]-thymidine incorporation (CPM)	100	1000	100	4200

The combination of flt3-L and IL-7 produced a response that was approximately four-fold greater than flt3-L alone and approximately 40-fold greater than IL-7 alone.

TABLE II

Effect of Flt3-L and IL-3 on Proliferation of C-*kit*+ Cells.

	Factor			
	Control (vector alone)	flt3-L	IL-3	flt3-L + IL-3
[^{3}H]-thymidine incorporation (CPM)	100	1800	3000	9100

Culture supernatant from CV1/EBNA cells transfected with flt3-L cDNA stimulated the proliferation of c-*kit*$^+$ stem cells approximately 18-fold greater than the culture supernatant of CV1/EBNA cells transfected with the expression vector alone. Addition of IL-3 to flt3-L containing supernatant showed a synergistic effect, with approximately twice the degree of proliferation observed than would be expected if the effects were additive.

EXAMPLE 8

Construction of Flt3-L:Fc Fusion Protein

This example describes a methof for constructing a fusion protein comprising an extracellular region of the flt3-L and the Fc domain of a human immunoglobulin. The methods are essentially the same as those described in Example 1 for construction of a flt3:Fc fusion protein.

Prior to fusing a flt3-L cDNA to the N-terminus of cDNA encoding the Fc portion of a human IgG1 molecule, the flt3-L cDNA fragment is inserted into Asp718-

*Not*I site of pCAV/NOT, described in PCT Application WO 90/05183. DNA encoding a single chain polypeptide comprising the Fc region of a human IgG1 antibody is cloned into the *Spe*I site of the pBLUESCRIPT SK® vector, which is commercially available from Stratagene Cloning Systems, La Jolla, California. This plasmid vector is replicable in *E. coli* and contains a polylinker segment that includes 21 unique restriction sites. A unique *Bgl*II site is then introduced near the 5' end of the inserted Fc encoding sequence, such that the *Bgl*II site encompasses the codons for amino acids three and four of the Fc polypeptide.

The encoded Fc polypeptide extends from the N-terminal hinge region to the native C-terminus, i.e., is an essentially full-length antibody Fc region. Fragments of Fc regions, e.g., those that are truncated at the C-terminal end, also may be employed. The fragments preferably contain multiple cysteine residues (at least the cysteine residues in the hinge reaction) to permit interchain disulfide bonds to form between the Fc polypeptide portions of two separate flt3-L:Fc fusion proteins, forming dimers.

An Asp718-*Stu*I partial cDNA of flt3-L in pCAV/NOT can be cloned into a Asp718-*Spe*I site of pBLUESCRIPT SK® vector containing the Fc cDNA, such that the flt3-L cDNA is positioned upstream of the Fc cDNA. The sequence of single stranded DNA derived from the resulting gene fusion can be affected by template-directed mutagensis described by Kunkel (*Proc. Natl. Acad. Sci. USA* 82:488, 1985) and Kunkel et al. (*Methods in Enzymol.* 154:367, 1987) in order to perfectly fuse the entire extracellular domain of flt3-L to the Fc sequence. The resulting DNA can then be sequenced to confirm that the proper nucleotides are removed (i.e., transmembrane region and partial cytoplasmic domain DNA are deleted) and that flt3-L and Fc sequences are in the same reading frame. The fusion cDNA is then excised and inserted using conventional methods into the mammalian expression vector pCAV/NOT which is cut with Asp 718-NotI.

Flt3-L:Fc fusion proteins preferably are synthesized in recombinant mammalian cell culture. The flt3-L:Fc fusion-containing expression vector is then transfected into CV-1 cells (ATCC CCL 70) or COS-7 cells (ATCC CRL 1651). Expression in 293 cells (transformed primary human embryonal kidney cells, ATCC CRL 1573) also is feasible.

The 293 cells transfected with the pCAV/NOT/flt3-L:Fc vector are cultivated in roller bottles to allow transient expression of the fusion protein, which is secreted into

the culture medium *via* the flt3-L signal peptide. The fusion protein can be purified on protein A Sepharose columns.

EXAMPLE 9

Generation of Transgenic Mice That Overexpress Flt3-L

This example describes a procedure used to generate transgenic mice that overexpress flt3-L. Flt3-L-overexpressing transgenic mice were studied to determine the biological effects of overexpression. Mouse (B16/J) pronuclei were microinjected with flt3-L DNA according to the method described by Gordon et al., *Science* 214:1244-1246, (1981). In general, fertilized mouse eggs having visible pronuclei were first placed on an injection chamber and held in place with a small pipet. An injection pipet was then used to inject the gene encoding the flt3-L (clone #6C) into the pronuclei of the egg. Injected eggs were then either (i) transferred into the oviduct of a 0.5 day p.c. pseudopregnant female; (ii) cultured *in vitro* to the two-cell stage (overnight) and transferred into the oviduct of a 0.5 day p.c. pseudopregnant female; or (iii) cultured *in vitro* to the blastocyst stage and transferred into the uterus of a 2.5 day p.c. pseudopregnant female. Preferably, either of the first two options can be used since they avoid extended *in vitro* culture, and preferably, approximately 20-30 microinjected eggs should be transferred to avoid small litters.

EXAMPLE 10

Flt3-L Stimulates Proliferation of Erythroid Cells in the Spleen

This example describes the effect of flt3-L on the production of erythroid cells in the spleen of transgenic mice. Transgenic mice were generated according to the procedures of Example 10. The mice were sacrificed and each intact spleen was made into a single cell suspension. The suspended cells were spun and then resuspended in 10 ml of medium that contained PBS + 1% fetal bovine serum. Cell counts were performed thereon using a hemocytometer. Each cell specimen was counted with Trypan Blue stain to obtain a total viable cell count per milliliter of medium according to the following formula: (RBC + WBC)/ ml, wherein RBC is the red blood cell count and WBC means the white blood cell count. Each specimen then was counted with Turk's stain to obtain a total white blood cell count per milliliter of medium. The total red blood cell count per milliliter was calculated for each specimen by subtracting the total white blood cell count per milliliter from the total viable cell count per milliliter. The results are shown in the following Table III.

TABLE III

Erythroid Cell Proliferation in Flt3-L-Overexpressing Transgenic Mice Spleen

Mouse	Total Viable Cell (million cells/ml)	Total White Cell (million cells/ml)	Total Red Blood Cell (million cells/ml)
Control 1	29.7	27	2.7
Control 2	31	24.6	6.4
Transgenic 1	44.7	25.6	19.1
Transgenic 2	37.3	28.4	8.9

From the data of Table III, the white blood cell counts per milliliter were approximately the same as the control mice. However, the red blood cell counts from the spleens of the two transgenic mice were approximately two to three-fold greater than observed in the control mice. Flt3-L stimulates an increase in cells of the erythroid lineage, possibly through stimulation of erythroid proogenitor cells, through the stimulation of cells that produce erythropoietin, or by blocking a mechanism that inhibits erythropoiesis.

EXAMPLE 11

Flt3-L Stimulates Proliferation of T Cells and Early B Cells

Bone marrow from 9 week old transgenic mice generated according to Example 10 was screened for the presence of various T and B cell phenotype markers using antibodies that are immunoreactive with such markers. The following markers were investigated: the B220 marker, which is specific to the B cell lineage; surface IgM marker (sIgM), which is specific to mature B cells; the S7 (CD43) marker, which is an early B cell marker; the Stem Cell Antigen-1 (SCA-1) marker, which is a marker of activated T cells and B cells; CD4, which is a marker for helper T cells and some stem cells; and the Mac-1 marker, which is specific to macrophages, were screened using well known antibodies against such markers. The following Table IV shows the data obtained from screening the bone marrow. Two transgenic mice from the same litter were analyzed against a normal mouse from the same litter (control), and an unrelated normal mouse (control).

TABLE IV

Effect of flt3-L Overexpression in Transgenic Mice

	Percentage of Positive Cells			
Marker	Unrelated Control	Littermate Control	Transgenic #1	Transgenic #2
B220	30.64	27.17	45.84	48.78
sIgM	3.54	2.41	1.94	1.14
S7(CD43)	54.43	45.44	46.11	50.59
SCA-1	10.92	11.74	19.45	27.37
CD4	6.94	8.72	12.21	14.05
Mac-1	36.80	27.15	21.39	18.63

The above data indicate that flt3-L overexpression in mice leads to an increase in the number of B cells, as indicated by the increase $B220^+$ cells and SCA-1^+ cells. Analysis of $B220^+$ cells by FACS indicated an increase in proB cells (HSA^-, $S7^+$). The increase in $CD4^+$ cells indicated an approximate two-fold increase in T cells and stem cells. The decrease in cells having the sIgM marker indicated that flt3-L does not stimulate proliferation of mature B cells. These data indicate that flt3-L increases cells with a stem cell, T cell or an early B cell phenotype, and does not stimulate proliferation of mature B cells or macrophages.

EXAMPLE 12

Analysis of the Thymus From Flt3-L-Over-expressing Mice

This Example describes the analysis of the thymus from the transgenic mice generated according to the procedure of Example 10. Six adult mice, each approximately three months of age, were sacrificed. The thymus from each mouse was removed and a single cell suspension was made.

FACS analysis demonstrated that no total change in cell number occurred and that the mice showed no change in the ratios of maturing thymocytes using the markers: CD4 vs. CD8; CD3 vs. αßTCR (T cell receptor); and CD3 vs. γδTCR (T cell receptor). However, a change in the ratios of certain cell types within the $CD4^-$ and $CD8^-$ compartment (i.e., the earliest cells with respect to development; which represent approximately 2% to 3% of total thymus cells) occurred. Specifically, $CD4^-$ and $CD8^-$ cells in the thymus develop in three stages. Stage 1 represents cells having the Pgp-1^{++}, HSA^+ and IL-2 receptor-negative ("IL-$2R^-$") markers. After stage 1, thymic cells develop to stage 2 consisting of cells having Pgp-1^+, HSA^{++}, and IL-$2R^{++}$ markers, and then to stage 3, characterized by cells having Pgp-$1^{+/-}$, HSA^{++}, and IL-$2R^-$

markers. Thymic cells in stage 2 of the transgenic mice were reduced by about 50%, while the population of cells in stage 3 was proportionately increased. These data suggest that flt3-L drives the thymic cells from stage 2 to stage 3 of development, indicating that flt3-L is active on early T cells.

EXAMPLE 13

Use of Flt3-L in Peripheral Stem Cell Transplantation

This Example describes a method for using flt3-L in autologous peripheral stem cell (PSC) or peripheral blood progenitor cell (PBPC) transplantation. Typically, PBPC and PSC transplantation is performed on patients whose bone marrow is unsuitable for collection due to, for example, marrow abnormality or malignant involvement.

Prior to cell collection, it may be desirable to mobilize or increase the numbers of circulating PBPC and PSC. Mobilization can improve PBPC and PSC collection, and is achievable through the intravenous administration of flt3-L to the patients prior to collection of such cells. Other growth factors such as CSF-1, GM-CSF, SF, G-CSF, EPO, IL-1, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-11, IL-12, IL-13, IL-14, IL-15, GM-CSF/IL-3 fusion proteins, LIF, FGF and combinations thereof, can be likewise administered in sequence, or in concurrent combination with flt3-L. Mobilized or non-mobilized PBPC and PSC are collected using apheresis procedures known in the art. See, for example, Bishop et al., *Blood*, vol. 83, No. 2, pp. 610-616 (1994). Briefly, PBPC and PSC are collected using conventional devices, for example, a Haemonetics Model V50 apheresis device (Haemonetics, Braintree, MA). Four-hour collections are performed typically no more than five times weekly until approximately 6.5 x 10^8 mononuclear cells (MNC)/kg patient are collected. Aliquots of collected PBPC and PSC are assayed for granulocyte-macrophage colony-forming unit (CFU-GM) content by diluting approximately 1:6 with Hank's balanced salt solution without calcium or magnesium (HBSS) and layering over lymphocyte separation medium (Organon Teknika, Durham, North Carolina). Following centrifugation, MNC at the interface are collected, washed and resuspended in HBSS. One milliliter aliquots containing approximately 300,000 MNC, modified McCoy's 5A medium, 0.3% agar, 200 U/mL recombinant human GM-CSF, 200 u/mL recombinant human IL-3, and 200 u/mL recombinant human G-CSF are cultured at 37 °C in 5% CO_2 in fully humidified air for 14 days. Optionally, flt3-L or GM-CSF/IL-3 fusion molecules (PIXY 321) may be added to the cultures. These cultures are stained with Wright's

stain, and CFU-GM colonies are scored using a dissecting microscope (Ward et al., *Exp. Hematol.*, 16:358 (1988). Alternatively, CFU-GM colonies can be assayed using the CD34/CD33 flow cytometry method of Siena et al., *Blood*, Vol. 77, No. 2, pp 400-409 (1991), or any other method known in the art.

CFU-GM containing cultures are frozen in a controlled rate freezer (e.g., Cryo-Med, Mt. Clemens, MI), then stored in the vapor phase of liquid nitrogen. Ten percent dimethylsulfoxide can be used as a cryoprotectant. After all collections from the patient have been made, CFU-GM containing cultures are thawed and pooled. The thawed cell collection either is reinfused intravenoulsy to the patient or expanded *ex vivo* prior to reinfusion. *Ex vivo* expansion of pooled cells can be performed using flt3-L as a growth factor either alone, sequentially or in concurrent combination with other cytokines listed above. Methods of such *ex vivo* expansion are well known in the art. The cells, either expanded or unexpanded, are reinfused intravenously to the patient. To facilitate engraftment of the transplanted cells, flt3-L is administered simultaneously with, or subsequent to, the reinfusion. Such administration of flt3-L is made alone, sequentially or in concurrent combination with other cytokines selected from the list above.

EXAMPLE 14

Purification of Hematopoietic Progenitor and Stem Cells Using Flt3-L

This Example describes a method for purifying hematopoietic progenitor cells and stem cells from a suspension containing a mixture of cells. Cells from bone marrow and peripheral blood are collected using conventional procedures. The cells are suspended in standard media and then centrifuged to remove red blood cells and neutrophils. Cells located at the interface between the two phases (also known in the art as the buffy coat) are withdrawn and resuspended. These cells are predominantly mononuclear and represent a substantial portion of the early hematopoietic progenitor and stem cells. The resulting cell suspension then is incubated with biotinylated flt3-L for a sufficient time to allow substantial flt3:flt3-L interaction. Typically, incubation times of at least one hour are sufficient. After incubation, the cell suspension is passed, under the force of gravity, through a column packed with avidin-coated beads. Such columns are well known in the art, see Berenson, et al., *J. Cell Biochem.*, 10D:239 (1986). The column is washed with a PBS solution to remove unbound material. Target cells can be released from the beads and from flt3-L using conventional methods.

SEQUENCE LISTING

(1) GENERAL INFORMATION:
(i) APPLICANT: Lyman, Stewart D.
Beckmann, M. Patricia

(ii) TITLE OF INVENTION: Ligands for flt3 Receptors

(iii) NUMBER OF SEQUENCES: 8

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(v) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: Apple Macintosh
(C) OPERATING SYSTEM: Macintosh 7.0.1
(D) SOFTWARE: Microsoft Word, Version #5.1

(vi) CURRENT APPLICATION DATA:
(A) APPLICATION NUMBER: -to be assigned-
(B) FILING DATE: March 7, 1994
(C) CLASSIFICATION:

(vii) PRIOR APPLICATION DATA:
(A) APPLICATION NUMBER: 08/162,407
(B) FILING DATE: December 3, 1993
(C) CLASSIFICATION:

(vii) PRIOR APPLICATION DATA:
(A) APPLICATION NUMBER: 08/111,758
(B) FILING DATE: August 25, 1993
(C) CLASSIFICATION:

(vii) PRIOR APPLICATION DATA:
(A) APPLICATION NUMBER: 08/106,463
(B) FILING DATE: August 12, 1993
(C) CLASSIFICATION:

(vii) PRIOR APPLICATION DATA:
(A) APPLICATION NUMBER: 08/068,394
(B) FILING DATE: May 24, 1993
(C) CLASSIFICATION:

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(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 879 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA to mRNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(ix) FEATURE:
(A) NAME/KEY: misc_feature
(B) LOCATION: 1..25

(ix) FEATURE:
(A) NAME/KEY: misc_feature
(B) LOCATION: 855..879

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 57..752

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

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GTCGACTGGA ACGAGACGAC CTGCTCTGTC ACAGGCATGA GGGGTCCCCG GCAGAG      56

ATG ACA GTG CTG GCG CCA GCC TGG AGC CCA AAT TCC TCC CTG TTG CTG   104
Met Thr Val Leu Ala Pro Ala Trp Ser Pro Asn Ser Ser Leu Leu Leu
 1               5                  10                  15

CTG TTG CTG CTG CTG AGT CCT TGC CTG CGG GGG ACA CCT GAC TGT TAC   152
Leu Leu Leu Leu Leu Ser Pro Cys Leu Arg Gly Thr Pro Asp Cys Tyr
            20                  25                  30
TTC AGC CAC AGT CCC ATC TCC TCC AAC TTC AAA GTG AAG TTT AGA GAG   200
Phe Ser His Ser Pro Ile Ser Ser Asn Phe Lys Val Lys Phe Arg Glu
        35                  40                  45

TTG ACT GAC CAC CTG CTT AAA GAT TAC CCA GTC ACT GTG GCC GTC AAT   248
Leu Thr Asp His Leu Leu Lys Asp Tyr Pro Val Thr Val Ala Val Asn
    50                  55                  60

CTT CAG GAC GAG AAG CAC TGC AAG GCC TTG TGG AGC CTC TTC CTA GCC   296
Leu Gln Asp Glu Lys His Cys Lys Ala Leu Trp Ser Leu Phe Leu Ala
65                  70                  75                  80

CAG CGC TGG ATA GAG CAA CTG AAG ACT GTG GCA GGG TCT AAG ATG CAA   344
Gln Arg Trp Ile Glu Gln Leu Lys Thr Val Ala Gly Ser Lys Met Gln
                85                  90                  95

ACG CTT CTG GAG GAC GTC AAC ACC GAG ATA CAT TTT GTC ACC TCA TGT   392
Thr Leu Leu Glu Asp Val Asn Thr Glu Ile His Phe Val Thr Ser Cys
            100                 105                 110
```

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ACC TTC CAG CCC CTA CCA GAA TGT CTG CGA TTC GTC CAG ACC AAC ATC   440
Thr Phe Gln Pro Leu Pro Glu Cys Leu Arg Phe Val Gln Thr Asn Ile
        115                 120                 125

TCC CAC CTC CTG AAG GAC ACC TGC ACA CAG CTG CTT GCT CTG AAG CCC   488
Ser His Leu Leu Lys Asp Thr Cys Thr Gln Leu Leu Ala Leu Lys Pro
    130                 135                 140

TGT ATC GGG AAG GCC TGC CAG AAT TTC TCT CGG TGC CTG GAG GTG CAG   536
Cys Ile Gly Lys Ala Cys Gln Asn Phe Ser Arg Cys Leu Glu Val Gln
145                 150                 155                 160

TGC CAG CCG GAC TCC TCC ACC CTG CTG CCC CCA AGG AGT CCC ATA GCC   584
Cys Gln Pro Asp Ser Ser Thr Leu Leu Pro Pro Arg Ser Pro Ile Ala
                165                 170                 175

CTA GAA GCC ACG GAG CTC CCA GAG CCT CGG CCC AGG CAG CTG TTG CTC   632
Leu Glu Ala Thr Glu Leu Pro Glu Pro Arg Pro Arg Gln Leu Leu Leu
            180                 185                 190

CTG CTG CTG CTG CTG CCT CTC ACA CTG GTG CTG CTG GCA GCC GCC TGG   680
Leu Leu Leu Leu Leu Pro Leu Thr Leu Val Leu Leu Ala Ala Ala Trp
        195                 200                 205

GGC CTT CGC TGG CAA AGG GCA AGA AGG AGG GGG GAG CTC CAC CCT GGG   728
Gly Leu Arg Trp Gln Arg Ala Arg Arg Arg Gly Glu Leu His Pro Gly
    210                 215                 220

GTG CCC CTC CCC TCC CAT CCC TAGGATTCGA GCCTTGTGCA TCGTTGACTC      779
Val Pro Leu Pro Ser His Pro
225                 230

AGCCAGGGTC TTATCTCGGT TACACCTGTA ATCTCAGCCC TTGGGAGCCC AGAGCAGGAT 839

TGCTGAATGG TCTGGAGCAG GTCGTCTCGT TCCAGTCGAC                       879
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(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 231 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

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Met Thr Val Leu Ala Pro Ala Trp Ser Pro Asn Ser Ser Leu Leu Leu
 1               5                  10                  15

Leu Leu Leu Leu Leu Ser Pro Cys Leu Arg Gly Thr Pro Asp Cys Tyr
            20                  25                  30

Phe Ser His Ser Pro Ile Ser Ser Asn Phe Lys Val Lys Phe Arg Glu
        35                  40                  45

Leu Thr Asp His Leu Leu Lys Asp Tyr Pro Val Thr Val Ala Val Asn
    50                  55                  60
```

```
Leu Gln Asp Glu Lys His Cys Lys Ala Leu Trp Ser Leu Phe Leu Ala
65                  70                  75                  80

Gln Arg Trp Ile Glu Gln Leu Lys Thr Val Ala Gly Ser Lys Met Gln
                85                  90                  95

Thr Leu Leu Glu Asp Val Asn Thr Glu Ile His Phe Val Thr Ser Cys
            100                 105                 110

Thr Phe Gln Pro Leu Pro Glu Cys Leu Arg Phe Val Gln Thr Asn Ile
        115                 120                 125

Ser His Leu Leu Lys Asp Thr Cys Thr Gln Leu Leu Ala Leu Lys Pro
    130                 135                 140

Cys Ile Gly Lys Ala Cys Gln Asn Phe Ser Arg Cys Leu Glu Val Gln
145                 150                 155                 160

Cys Gln Pro Asp Ser Ser Thr Leu Leu Pro Pro Arg Ser Pro Ile Ala
                165                 170                 175

Leu Glu Ala Thr Glu Leu Pro Glu Pro Arg Pro Arg Gln Leu Leu Leu
            180                 185                 190

Leu Leu Leu Leu Leu Pro Leu Thr Leu Val Leu Leu Ala Ala Ala Trp
        195                 200                 205

Gly Leu Arg Trp Gln Arg Ala Arg Arg Arg Gly Glu Leu His Pro Gly
    210                 215                 220

Val Pro Leu Pro Ser His Pro
225                 230
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(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 24 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

TCGACTGGAA CGAGACGACC TGCT 24

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

AGCAGGTCGT CTCGTTCCAG 20

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 988 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA to mRNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 30..734

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

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CGGCCGGAAT TCCGGGGCCC CCGGCCGAA ATG ACA GTG CTG GCG CCA GCC TGG    53
                                Met Thr Val Leu Ala Pro Ala Trp
                                 1               5

AGC CCA ACA ACC TAT CTC CTC CTG CTG CTG CTG CTG AGC TCG GGA CTC  101
Ser Pro Thr Thr Tyr Leu Leu Leu Leu Leu Leu Leu Ser Ser Gly Leu
     10                  15                  20

AGT GGG ACC CAG GAC TGC TCC TTC CAA CAC AGC CCC ATC TCC TCC GAC  149
Ser Gly Thr Gln Asp Cys Ser Phe Gln His Ser Pro Ile Ser Ser Asp
 25                  30                  35                  40

TTC GCT GTC AAA ATC CGT GAG CTG TCT GAC TAC CTG CTT CAA GAT TAC  197
Phe Ala Val Lys Ile Arg Glu Leu Ser Asp Tyr Leu Leu Gln Asp Tyr
                 45                  50                  55

CCA GTC ACC GTG GCC TCC AAC CTG CAG GAC GAG GAG CTC TGC GGG GGC  245
Pro Val Thr Val Ala Ser Asn Leu Gln Asp Glu Glu Leu Cys Gly Gly
             60                  65                  70

CTC TGG CGG CTG GTC CTG GCA CAG CGC TGG ATG GAG CGG CTC AAG ACT  293
Leu Trp Arg Leu Val Leu Ala Gln Arg Trp Met Glu Arg Leu Lys Thr
         75                  80                  85

GTC GCT GGG TCC AAG ATG CAA GGC TTG CTG GAG CGC GTG AAC ACG GAG  341
Val Ala Gly Ser Lys Met Gln Gly Leu Leu Glu Arg Val Asn Thr Glu
     90                  95                  100

ATA CAC TTT GTC ACC AAA TGT GCC TTT CAG CCC CCC CCC AGC TGT CTT  389
Ile His Phe Val Thr Lys Cys Ala Phe Gln Pro Pro Pro Ser Cys Leu
105                 110                 115                 120
```

```
CGC TTC GTC CAG ACC AAC ATC TCC CGC CTC CTG CAG GAG ACC TCC GAG   437
Arg Phe Val Gln Thr Asn Ile Ser Arg Leu Leu Gln Glu Thr Ser Glu
                125                 130                 135

CAG CTG GTG GCG CTG AAG CCC TGG ATC ACT CGC CAG AAC TTC TCC CGG   485
Gln Leu Val Ala Leu Lys Pro Trp Ile Thr Arg Gln Asn Phe Ser Arg
            140                 145                 150

TGC CTG GAG CTG CAG TGT CAG CCC GAC TCC TCA ACC CTG CCA CCC CCA   533
Cys Leu Glu Leu Gln Cys Gln Pro Asp Ser Ser Thr Leu Pro Pro Pro
        155                 160                 165

TGG AGT CCC CGG CCC CTG GAG GCC ACA GCC CCG ACA GCC CCG CAG CCC   581
Trp Ser Pro Arg Pro Leu Glu Ala Thr Ala Pro Thr Ala Pro Gln Pro
    170                 175                 180

CCT CTG CTC CTC CTA CTG CTG CTG CCC GTG GGC CTC CTG CTG CTG GCC   629
Pro Leu Leu Leu Leu Leu Leu Leu Pro Val Gly Leu Leu Leu Leu Ala
185                 190                 195                 200

GCT GCC TGG TGC CTG CAC TGG CAG AGG ACG CGG CGG AGG ACA CCC CGC   677
Ala Ala Trp Cys Leu His Trp Gln Arg Thr Arg Arg Arg Thr Pro Arg
                205                 210                 215

CCT GGG GAG CAG GTG CCC CCC GTC CCC AGT CCC CAG GAC CTG CTG CTT   725
Pro Gly Glu Gln Val Pro Pro Val Pro Ser Pro Gln Asp Leu Leu Leu
            220                 225                 230

GTG GAG CAC TGACCTGGCC AAGGCCTCAT CCTGCGGAGC CTTAAACAAC          774
Val Glu His
        235

GCAGTGAGAC AGACATCTAT CATCCCATTT TACAGGGGAG GATACTGAGG CACACAGAGG 834

GGAGTCACCA GCCAGAGGAT GTATAGCCTG GACACAGAGG AAGTTGGCTA GAGGCCGGTC 894

CCTTCCTTGG GCCCCTCTCA TTCCCTCCCC AGAATGGAGG CAACGCCAGA ATCCAGCACC 954

GGCCCCATTT ACCCAACTCT GAACAAAGCC CCCG                             988
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(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 235 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

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Met Thr Val Leu Ala Pro Ala Trp Ser Pro Thr Thr Tyr Leu Leu Leu
  1               5                  10                  15

Leu Leu Leu Leu Ser Ser Gly Leu Ser Gly Thr Gln Asp Cys Ser Phe
            20                  25                  30

Gln His Ser Pro Ile Ser Ser Asp Phe Ala Val Lys Ile Arg Glu Leu
        35                  40                  45
```

```
Ser Asp Tyr Leu Leu Gln Asp Tyr Pro Val Thr Val Ala Ser Asn Leu
     50                  55                  60

Gln Asp Glu Glu Leu Cys Gly Gly Leu Trp Arg Leu Val Leu Ala Gln
 65                  70                  75                  80

Arg Trp Met Glu Arg Leu Lys Thr Val Ala Gly Ser Lys Met Gln Gly
                 85                  90                  95

Leu Leu Glu Arg Val Asn Thr Glu Ile His Phe Val Thr Lys Cys Ala
            100                 105                 110

Phe Gln Pro Pro Pro Ser Cys Leu Arg Phe Val Gln Thr Asn Ile Ser
        115                 120                 125

Arg Leu Leu Gln Glu Thr Ser Glu Gln Leu Val Ala Leu Lys Pro Trp
    130                 135                 140

Ile Thr Arg Gln Asn Phe Ser Arg Cys Leu Glu Leu Gln Cys Gln Pro
145                 150                 155                 160

Asp Ser Ser Thr Leu Pro Pro Pro Trp Ser Pro Arg Pro Leu Glu Ala
                165                 170                 175

Thr Ala Pro Thr Ala Pro Gln Pro Pro Leu Leu Leu Leu Leu Leu Leu
            180                 185                 190

Pro Val Gly Leu Leu Leu Leu Ala Ala Ala Trp Cys Leu His Trp Gln
        195                 200                 205

Arg Thr Arg Arg Arg Thr Pro Arg Pro Gly Glu Gln Val Pro Pro Val
    210                 215                 220

Pro Ser Pro Gln Asp Leu Leu Leu Val Glu His
225                 230                 235
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(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 71 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA to mRNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

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AATTGGTACC TTTGGATAAA AGAGACTACA AGGACGACGA TGACAAGACA CCTGACTGTT  60

ACTTCAGCCA C                                                      71
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(2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 37 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA to mRNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

ATATGGATCC CTACTGCCTG GGCCGAGGCT CTGGGAG 37

What is claimed is:

1. An isolated flt3-ligand (flt3-L) polypeptide.
2. A polypeptide according to claim 1 that is murine flt3-L.
3. A polypeptide according to claim 1 that is human flt3-L.
4. A polypeptide according to claim 3, comprising amino acids 1-235 of SEQ ID NO:6.
5. A polypeptide according to claim 1 that is a soluble flt3-L.
6. A polypeptide according to claim 5, comprising amino acids 28-160 or 28-182 of SEQ ID NO:6.
7. A polypeptide according to claim 3 that is encoded by the cDNA insert of vector sfHAVEO410 in *E. coli* DH10B cells having accession number ATCC 69382.
8. An isolated DNA sequence encoding a flt3-L polypeptide.
9. An isolated DNA sequence according to claim 8, encoding a murine flt3-L polypeptide.
10. An isolated DNA sequence according to claim 8, encoding a human flt3-L polypeptide.
11. An isolated DNA sequence according to claim 8, which encodes the amino acid sequence 28-160 or 28-182 of SEQ ID NO:6.
12. A DNA according to claim 8, selected from the group consisting of:
    (a) cDNA derived from the coding region of a flt3-L gene;
    (b) cDNA sequences selected from the group consisting of SEQ ID NO:1 and SEQ ID NO:5;
    (c) DNA sequences that hybridize under moderately stringent conditions to the cDNA of (a) or (b), and which DNA sequences encode flt3-L;
    (d) DNA sequences that, due to the degeneracy of the genetic code, encode flt3-L polypeptides having the animo acid sequence of the polypeptides encoded by the DNA sequences of (a), (b) or (c).
13. An expression vector comprising a DNA sequence according to claim 8.
14. An expression vector comprising a DNA sequence according to claim 9.
15. An expression vector comprising a DNA sequence according to claim 10.
16. An expression vector comprising a DNA sequence according to claim 11.
17. An expression vector comprising a DNA sequence according to claim 12.
18. A host cell transfected or transformed with the expression vector according to claim 13.

19. A host cell transformed or transfected with the expression vector according to claim 14.
20. A host cell transformed or transfected with the expression vector according to claim 15.
21. A host cell transformed or transfected with the expression vector according to claim 16.
22. A host cell transformed or transfected with the expression vector according to claim 17.
23. A process for producing a flt3-L polypeptide, comprising culturing a host cell according to claim 18 under conditions promoting expression, and recovering the polypeptide from the culture medium.
24. A process for producing a flt3-L polypeptide, comprising culturing a host cell according to claim 19 under conditions promoting expression, and recovering the polypeptide from the culture medium.
25. A process for producing a flt3-L polypeptide, comprising culturing a host cell according to claim 20 under conditions promoting expression, and recovering the polypeptide from the culture medium.
26. A process for producing a flt3-L polypeptide, comprising culturing a host cell according to claim 21 under conditions promoting expression, and recovering the polypeptide from the culture medium.
27. A process for producing a flt3-L polypeptide, comprising culturing a host cell according to claim 22 under conditions promoting expression, and recovering the polypeptide from the culture medium.
28. An antibody that is immunoreactive with a flt3-L polypeptide.
29. An antibody according to claim 28 that is a monoclonal antibody.
30. A pharmaceutical composition comprising an effective amount of a flt3-L polypeptide according to claim 1 and a pharmaceutically acceptable carrier, excipient or diluent.
31. A pharmaceutical composition comprising an effective amount of a flt3-L polypeptide according to claim 3 and a pharmaceutically acceptable carrier, excipient or diluent.
32. A pharmaceutical composition comprising an effective amount of a flt3-L polypeptide according to claim 5 and a pharmaceutically acceptable carrier, excipient or diluent.
33. A method for conducting autologous transplantation in a patient receiving cytoreductive therapy, comprising:

(a) collecting hematopoietic progenitor cells or stem cells from the patient prior to cytoreductive therapy; and

(b) administering the collected cells to the patient following cytoreductive therapy;

wherein the method further comprises at least one of the following steps:

(i) administering an effective amount of flt3-L to the patient to increase the number of circulating progenitor cells or stem cells prior to collection;

(ii) expanding the progenitor cells or stem cells *ex vivo* by contacting them with an effective amount of flt3-L; and

(iii) administering an effective amount of flt3-L to the patient to facilitate engraftment of the transplanted progenitor or stem cells in the patient.

34. A method according to claim 33, wherein flt3-L is used in combination with a cytokine selected from the group consisting of CSF-1, GM-CSF, SF, G-CSF, EPO, IL-1, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-11, IL-12, IL-13, IL-14, IL-15, GM-CSF/IL-3 fusion proteins, LIF and FGF, and sequential or concurrent combinations thereof.

35. A method according to claim 34, wherein flt3-L is used in combination with a cytokine selected from the group consisting of GM-CSF, SF, G-CSF, EPO, IL-3 and GM-CSF/IL-3 fusion proteins.

36. A hematopoietic cell expansion media comprising cell growth media, and an effective amount of a flt3-L polypeptide according to claim 1.

37. A method of transfecting an exogenous gene into an early hematopoietic cell comprising the steps of:

(a) culturing the early hematopoietic cells in media comprising an effective amount of a flt3-L polypeptide; and

(b) transfecting the cultured cells from step (a) with the gene.

38. A method of transferring an exogenous gene to a mammal comprising the steps of:

(a) culturing early hematopoietic cells in media comprising an effective amount of a flt3-L polypeptide;

(b) transfecting the cultured cells from step (a) with the gene; and

(c) administering the transfected cells to the mammal.

39. A method of stimulating the proliferation of T cells in a mammal comprising administering to the mammal an effective amount of a flt3-L polypeptide according to claim 1.

40. A method of stimulating the proliferation of cells of the erythroid lineage in the spleen of a mammal comprising administering to the mammal an effective amount of a flt3-L polypeptide according to claim 1.

41. A method according to claim 40, further comprising the administration of an effective amount of EPO.

42. A method of treating a patient having symptoms of myelodysplastic syndrome, comprising the administration to the patient of an effective amount of a flt3-L polypeptide according to claim 1 and, optionally, an effective amount of one or more growth factors selected from the group consisting of CSF-1, GM-CSF, SF, G-CSF, EPO, IL-1, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-11, IL-12, IL-13, IL-14, IL-15, GM-CSF/IL-3 fusion proteins, LIF and FGF.

43. A method of treating a patient having symptoms of anemia, comprising the administration to the patient of an effective amount of a flt3-L polypeptide according to claim 1 and, optionally, an effective amount of one or more growth factors selected from the group consisting of CSF-1, GM-CSF, SF, G-CSF, EPO, IL-1, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-11, IL-12, IL-13, IL-14, IL-15, GM-CSF/IL-3 fusion proteins, LIF and FGF.

44. A method of treating a patient having symptoms of acquired immune deficiency syndrome, comprising the administration to the patient of an effective amount of a flt3-L polypeptide according to claim 1 and, optionally, an effective amount of one or more growth factors selected from the group consisting of CSF-1, GM-CSF, SF, G-CSF, EPO, IL-1, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-11, IL-12, IL-13, IL-14, IL-15, GM-CSF/IL-3 fusion proteins, LIF and FGF.

45. A method according to claim 44, wherein the patient is receiving AZT therapy.

46. A transgenic non-human mammal all of whose germ and somatic cells contain a DNA sequence according to claim 8 introduced into said mammal, or an ancestor of said mammal, at an embryonic stage.

47. A method of separating cells having the flt3 receptor on the surface thereof from a mixture of cells in suspension, comprising contacting the cells in the mixture with a contacting surface having a flt3-binding protein thereon, and separating the contacting surface and the suspension.

48. A method according to claim 47, wherein the flt3-binding protein is flt3-L.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US94/05365

A. CLASSIFICATION OF SUBJECT MATTER

IPC(5) :Please See Extra Sheet.
US CL :424/3, 93U; 435/69.1, 240.1, 252.3, 320.1, 240.3; 514/12, 44; 530.387,9; 536/23.5; 800/2

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 424/3, 93U; 435/69.1, 240.1, 252.3, 320.1, 240.3; 514/12, 44; 530.387,9; 536/23.5; 800/2

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, MEDLINE
search terms: flt3, flk2, hematopoietic stem cells, anemia, AZT, HIV, myelodysplasia

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X Y	US, A, 5,185,438 (LEMISCHKA) 9 February 1993, entire document.	1-32, 39-45, 47, 48 33-38,46
Y	Cell, Volume 63, issued 5 October 1990, J.G. Flanagan and P. Leder, "The kit Ligand: A cell surface molecule altered in steel mutant fibroblasts", pages 185-194, entire document.	1-27, 30-32
Y	Oncogene, Volume 6, issued 1991; O. Rosnet et al., "Murine Flt3, a gene encoding a novel tyrosine kinase receptor of the PDGFR/CSF1R family", pages 1641-1650, entire document.	1-48

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search 13 JULY 1994	Date of mailing of the international search report AUG 10 1994
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer LORRAINE M. SPECTOR Telephone No. (703) 308-0196

Form PCT/ISA/210 (second sheet)(July 1992)*

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US94/05365

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X,P	Cell, Volume 75, issued 17 December 1993, S.D. Lyman et al., "Molecular cloning of a ligand for the flt3/flk-2 tyrosine kinase receptor: a proliferative factor for primitive hematopoietic cells", pages 1157-1167, entire document.	1-32, 39-45, 47, 48
Y	US, A, 5,199,942 (GILLIS) 6 April 1993, see especially Abstract.	33-35, 37, 38
Y	US, A, 5,192,553 (BOYSE ET AL.) 9 March 1993, entire document, especially column 20 and Table 2, columns 27-29.	42-45, 47, 48
Y	US, A, 4,745,099 (AKAMATSU ET AL.) 17 May 1988, column 1 line 33 to column 2, line 26.	43
Y	US, A, 5,013,824 (ABRAMS ET AL.) 7 May 1991, column 2, lines 40-51.	43
Y	US,A, 5,057,420 (MASSEY) 15 October 1991, entire document.	46
Y	US, A, 5,114,710 (TAKAKU ET AL.) 19 May 1992, column 1 lines 40-48.	43
Y	Science, Volume 246, issued 8 December 1989, D. Hanahan, "Transgenic mice as probes into complex systems", pages 1265-1275, entire document.	46

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US94/05365

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

Please See Extra Sheet.

1. ☒ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US94/05365

A. CLASSIFICATION OF SUBJECT MATTER:
IPC (5):

G01N 1/34, 33/48; C12N 5/00, 1/21; A61K 37/02, 48/00; C07K 13/00, 15/28; C07H 21/04; C12N 15/12, 15/64; A01K 67/00

BOX II. OBSERVATIONS WHERE UNITY OF INVENTION WAS LACKING
This ISA found multiple inventions as follows:

This application contains the following inventions or groups of inventions which are not so linked as to form a single inventive concept under PCT Rule 13.1. In order for all inventions to be examined, the appropriate additional examination fees must be paid.

Group I, claims 1-27 and 30-32, drawn to flt3 ligand protein, DNA, vectors, transformed cells, and recombinant production of protein.

Group II, claims 28 and 29, drawn to anti flt3-ligand antibodies.

Group III, claims 33-35, drawn to a method for conducting autologous transplantation.

Group IV, claim 36, drawn to cell culture medium.

Group V, claim 37, drawn to a transfection method.

Group VI, claim 38, drawn to a gene therapy method.

Group VII, claims 39-45, drawn to an in vivo method of treatment to cause stem cell proliferation.

Group VIII, claim 46, drawn to transgenic animals.

Group IX, claims 47 and 48, drawn to a cell sorting method.

The inventions listed as Groups I-IX do not relate to a single inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons:

Group II is drawn to a product which is independent and distinct from the products of group I, and which is not linked by a special technical feature within the meaning of PCT Rule 13.2 to such products so as to form a single inventive concept. Groups III-IX are drawn to multiple methods of use of flt3 ligand or nucleic acids encoding such. These methods are independent and distinct and are not so linked by a special technical feature within the meaning of PCT Rule 13.2 so as to form a single inventive concept. Note that PCT Rule 13 does not provide for multiple methods within a single general inventive concept.